Preparation of Misacylated Aminoacyl-tRNA^{Phe}'s Useful as Probes of the Ribosomal Acceptor Site†

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ABSTRACT: Several pyroglutamylaminoacyl-tRNA's were prepared by T4 RNA ligase mediated condensation of synthetic pyroglutamylaminoacyl-pCpA's with tRNA's from which the last two nucleotides at the 3′-end had been removed. The derived pyroglutamylaminoacyl-tRNA's were incubated in the presence of calf liver pyroglutamate aminopeptidase, which effected their conversion to free aminoacyl-tRNA's. The lack of contaminating esterase activities in the pyroglutamate aminopeptidase was verified by direct assay for the presence of the aminoacyl moieties in the formed aminoacyl-tRNA's and by the use of the deblocked aminoacyl-tRNA's as acceptors in the peptidyltransferase reaction using an *Escherichia coli* ribosomal system. These findings provide the wherewithal for a detailed investigation of the substrate specificity of the peptidyltransferase center and for the elaboration of polypeptides containing modified amino acids at predetermined sites.

In ribosomal protein biosynthesizing systems, peptide bond formation occurs at the peptidyltransferase center (Allen & Zamecnik, 1962; Nathans, 1964; Traut & Monro, 1964; Monro, 1967; Harris & Symons, 1973a,b; Symons et al., 1979). In bacteria, ribosome mapping and biochemical studies have localized this center to the 50S ribosomal subunit and suggested that the constituent RNA's may play a role in this catalytic function (Cooperman, 1979; Krayevsky & Kukhanova, 1979; Noller, 1984; Hall et al., 1985; Lake, 1985). The chemical and structural features required for efficient participation of aminoacyl- and peptidyl-tRNA's in the peptidyltransferase reaction have been studied by the use of puromycin analogues (Nathans & Neidle, 1963), aminoacylated oligonucleotides structurally similar to the 3′-end of aminoacyl-tRNA's (Monro & Marcker, 1967; Monro et al., 1968; Mercer & Symons, 1972; Hecht, 1977; Quiggle et al., 1981), and aminoacyl-tRNA analogues obtained by aminoacyl-tRNA synthetase catalyzed production of structurally altered products [Hecht et al., 1974; Chinali et al., 1974; Hecht (1977) and references cited therein; Alford & Hecht, 1978; Sprinzl & Cramer, 1979; Pezzuto & Hecht, 1980; Wagner & Sprinzl, 1983] or by chemical alteration of normal aminoacyl-tRNA's (Chapeville et al., 1962; Fahnestock & Rich, 1971a,b).

A more generally applicable approach to the preparation of misacylated tRNA's, involving T4 RNA ligase mediated coupling of (protected) aminoacyl-pCpA derivatives to tRNA's lacking this dinucleotide at the 3′-end, has been developed in this laboratory (Heckler et al., 1984a,b) and used to prepare peptidyl-tRNA analogues that were assayed as donors in the ribosomal P-site in an in vitro protein biosynthesizing system (Heckler et al., 1983, 1988; Roesser et al., 1986). To date, all of the misacylated tRNA's prepared by this technique have been N-acylated aminoacyl-tRNA's of interest as analogues of peptidyl-tRNA's. To facilitate mechanistic dissection of events at the ribosomal A-site during peptide bond formation, we sought to extend our study of "chemically misacylated" tRNA's to include aminoacyl-tRNA's. Because free aminoacyl-tRNA's undergo facile solvolysis under the conditions employed for T4 RNA ligase mediated coupling of tRNA-C_{OH}[1] and aminoacyl-pCpA's, the latter have been protected chemically on N^{α} of the aminoacyl moiety to permit reasonable amounts of misacylated tRNA's to be produced (Heckler et al., 1984a,b). One logical approach to obtaining the requisite misacylated aminoacyl-tRNA's would involve the use of aminoacyl-pCpA derivatives chemically stabilized by introduction of a protecting group on N^{α} of the aminoacyl moiety; deblocking of the derived N-protected aminoacyl-tRNA's would then afford the aminoacyl-tRNA analogues of interest.

Reported herein is the chemical synthesis of several *N*-pyroglutamylaminoacyl-pCpA derivatives, their T4 RNA ligase mediated coupling to *Escherichia coli* tRNA^{Phe}-C_{OH}, and the successful deblocking of the derived *N*-pyroglutamyl-aminoacyl-tRNA^{Phe}'s with calf liver pyroglutamate aminopeptidase to provide misacylated tRNA^{Phe} derivatives without concomitant hydrolysis of the ester bond between the amino acid and tRNA. The generality of the deblocking procedure is also demonstrated for four other *E. coli* tRNA's containing their cognate amino acids. The utility of this approach is illustrated by the participation of the formed aminoacyl-tRNA^{Phe}'s as acceptors in dipeptide-forming reactions, including three species having amino acid analogues that do not normally participate in ribosomally mediated protein biosynthesis. To facilitate the application of this approach to the study of biochemical problems, we also describe a new, facile method for the preparation of tRNA-C_{OH} and improvements in the chemical synthesis of pCpA that provide this dinucleotide in greatly improved overall yield.

Experimental Procedures

Materials. Calf liver pyroglutamate aminopeptidase (1 unit is defined as the amount of enzyme that hydrolyzes 1 nmol

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[1] Abbreviations: tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; BD-cellulose, benzoylated (diethylaminoethyl)-cellulose; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; DEAE-cellulose, (diethylaminoethyl)-cellulose; THF, tetrahydrofuran; TTAB, tetradecyltrimethylammonium bromide.

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of L-pyroglutamic acid β-naphthylamide in 1 min at 37 °C), calf intestinal phosphatase (1 unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate in 1 min at 37 °C), *Escherichia coli* $tRNA^{Phe}$ (specific activity ~1300 pmol/A_{260} unit), and BD-cellulose were purchased from Boehringer Mannheim. DEAE-cellulose was purchased from Whatman. *E. coli* $tRNA^{Val}$, $tRNA^{Leu}$, $tRNA^{Tyr}$, and $tRNA^{Gly}$ were from Plenum Scientific Research. Poly(uridylic acid) (M_r >10^5), bovine serum albumin, L-pyroglutamic acid, and CpA were from Sigma Chemicals. T4 polynucleotide kinase was from U.S. Biochemicals; 1 unit is the amount of enzyme that incorporates 1 nmol of [α-^{32}P]ATP into micrococcal nuclease treated calf thymus DNA in 30 min at 37 °C. Bovine pancreatic ribonuclease A [3600 units/mg; 1 unit increased the absorbance of an RNA substrate by 1.0 A_{260} unit at 37 °C and pH 5.0 under defined conditions (Kalnitsky et al., 1959)] was purchased from Worthington Biochemicals. T4 RNA ligase was obtained from Pharmacia P-L Biochemicals; 1 unit was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphatase-resistant ^{32}P from [5′-^{32}P]oligo(rA)$_n$ in 30 min at 37 °C. [^{3}H]-L-Phenylalanine (37 Ci/mmol) was purchased from Amersham and purified by chromatography on BD-cellulose prior to use. [^{3}H]Tyrosine, [^{3}H]valine, [^{3}H]leucine, and [^{3}H]glycine were from ICN. Glass fiber disks were from Schleicher & Schuell.

E. coli aminoacyl-tRNA synthetases were isolated as described by Stulberg (1967); yeast phenylalanyl-tRNA synthetase was purified by the procedure of Roe et al. (1973). CTP(ATP):tRNA nucleotidyltransferase was purified as described (Alford et al., 1977) from yeast. *E. coli* ribosomes were prepared by the method of Pezzuto and Hecht (1980). *E. coli* *N*-acetyl[^{3}H]-L-phenylalanyl-$tRNA^{Phe}$ (~3 Ci/mmol) was prepared essentially as described (Rappoport & Lapidot, 1974) and purified by chromatography on BD-cellulose (Heckler et al., 1984a).

N-(Pyroglutamyloxy)succinimide. A solution of 2.3 g (20 mmol) of *N*-hydroxysuccinimide in 100 mL of ethyl acetate was treated with 4.13 g (20 mmol) of *N,N′*-dicyclohexylcarbodiimide and 2.6 g (20 mmol) of L-pyroglutamic acid. The reaction mixture was stirred at 25 °C for 8 h and then filtered to remove dicyclohexylurea. The filtrate was concentrated, and the crude product was crystallized from methanol as colorless microcrystals, yield 4.0 g (80%): mp 160 °C; ^{1}H NMR ([$^2H_6$]acetone) δ 2.65–3.20 (m, 8) and 3.70 (m, 1).

Preparation of 2′(3′)-O-(N-Pyroglutamylaminoacyl)pCpA's (**6c**). These compounds were prepared by acylation of a suitably protected pCpA derivative (Heckler et al., 1984a,b). The chemical synthesis of the latter has been improved significantly, as outlined below. Also described is an alternative synthesis of pCpA involving phosphorylation of commercially available CpA with polynucleotide kinase.

Synthesis of pCpA (**4**). *Method A.* A solution containing 1.57 g (2.15 mmol) of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(methoxytrityl)cytidine (**1**) (dried immediately prior to use by successive coevaporations of portions of dry pyridine and dry THF) in 15 mL of dry THF was added dropwise over a period of 30 min to a cold (−78 °C), stirred solution containing 200 μL (281 mg; 2.12 mmol) of freshly distilled methyl phosphorodichloridite (Martin & Pizzolato, 1950; Ogilvie et al., 1980) and 780 μL (715 mg; 5.90 mmol) of dry collidine in 8.5 mL of dry THF. The combined solution was stirred at −78 °C for 10 min and then treated dropwise over a period of 30 min with a solution containing 951 mg (1.64 mmol) of N^6,$O^{2′}$,$O^{3′}$-tribenzoyladenosine (**2**) (Ikehara et al., 1966) in 7.5 mL of dry THF. The combined solution was stirred at −78 °C for 15 min and then allowed to warm to 0 °C. The reaction mixture was then treated with 572 mg (2.25 mmol) of iodine in 20 mL of 2:2:1 THF–lutidine–H_2O and stirred for an additional 5 min. The solution was concentrated under diminished pressure, diluted with 100 mL of CH_2Cl_2, and extracted with aqueous $NaHSO_3$. The organic phase was dried (Na_2SO_4) and concentrated to afford a foam; residual collidine was removed by trituration with hexane. Flash chromatography (Still et al., 1978) was carried out on a 10 × 3 cm silica gel column. The column was washed with 2% CH_3OH in CH_2Cl_2, which effected elution of contaminants, and then with 7.5% CH_3OH in CH_2Cl_2, which provided methyl N^4-benzoyl-$O^{2′}$-(*tert*-butyldimethylsilyl)-$O^{5′}$-(methoxytrityl)cytidylyl(3′→5′)-N^6,$O^{2′}$,$O^{3′}$-tribenzoyladenosine (**3a**) as a colorless foam, yield 1.60 g (70%). This material was identical (TLC, UV) with a sample of **3a** prepared previously (Heckler et al., 1984a,b).

Detritylation was accomplished by dissolving 1.53 g (1.10 mmol) of **3a** in 15 mL of 2% trifluoroacetic acid in CH_2Cl_2 and stirring at 25 °C for 20 min. The reaction mixture was treated with 0.5 mL of methanol and stirred at 25 °C for an additional 60 min. The reaction mixture was neutralized with $NaHCO_3$ solution and partitioned between CH_2Cl_2 and water. The organic phase was dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 3 cm); elution with 4% CH_3OH in CH_2Cl_2 provided methyl N^4-benzoyl-$O^{2′}$-(*tert*-butyldimethylsilyl)cytidylyl(3′→5′)-N^6,$O^{2′}$,$O^{3′}$-tribenzoyladenosine (**3b**) as a white powder, yield 1.0 g (81%). This material was identical (TLC, UV, ^{1}H NMR) with a sample of **3b** prepared previously (Heckler et al., 1984a,b).

Phosphorylation of **3b** was carried out with 288 μL (474 mg; 3.0 mmol) of $POCl_3$ in 3.0 mL of collidine and 6 mL of THF at −78 °C. A solution containing 672 mg (0.60 mmol) of dinucleotide monophosphate **3b** in 6 mL of THF was added dropwise over a period of 20 min at −78 °C under N_2. The reaction mixture was stirred at −78 °C for 1 h and then at 0 °C for 30 min. The reaction mixture was then treated with 0.5 mL of H_2O, stirred at 0 °C for an additional 30 min, and concentrated to dryness. The residue was dissolved in 100 mL of $CHCl_3$, and the $CHCl_3$ solution was washed with water, dried (Na_2SO_4), and concentrated. The residue was treated with 5 mL of a 1:1:0.5 solution of dioxane–triethylamine–thiophenol, and the solution was maintained at 25 °C for 1 h. The solution was concentrated to dryness, and the residue was triturated with 10 mL of benzene and then dissolved in 50 mL of concentrated NH_4OH and stirred at 25 °C for 15 h. The solution was concentrated, and the residue was purified by chromatography on a (40 × 3 cm) silica gel column. Elution with 7:3:2 2-propanol–NH_4OH–H_2O afforded 2′-*O*-(*tert*-butyldimethylsilyl)cytidylyl(3′→5′)adenosine 5′-monophosphate as an off-white solid, yield 314 mg (68%). This compound was identical with a sample prepared previously (Heckler et al., 1984a,b) as judged by TLC, UV, and ^{1}H NMR properties.

The 2′-O-silylated pCpA was dissolved in 50 mL of a THF solution containing 1 M tetrabutylammonium fluoride. The reaction mixture was stirred at 25 °C for 5 h and then diluted with 500 mL of water and applied to a DEAE-cellulose column (30 × 3 cm, HCO_3^- form). The column was washed with 500 mL of water and then with 300 mL of 0.5 M aqueous ammonium bicarbonate. The bicarbonate elute was concentrated to a small volume and desalted by repeated evaporations of portions of water. The residue was purified by preparative silica gel TLC; development was with 7:3:2 2-propanol–

NH_4OH–H_2O. The major UV-active band was eluted from the plate with 9:1 CH_3OH–H_2O, providing cytidylyl(3′→5′)adenosine 5′-monophosphate (**4**; pCpA) as a white solid, yield 227 mg (85%). This synthetic material had the same properties (UV, TLC, ^1H NMR) as a sample of pCpA prepared previously (Heckler et al., 1984a,b).

Method B. A reaction mixture containing 500 μL (total volume) of 50 mM Tris-HCl, pH 7.6, 1.0 mg of cytidylyl(3′→5′)adenosine (CpA), 10 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM spermidine was treated with 10 units of T4 polynucleotide kinase and then incubated at 37 °C for 24 h. The reaction solution was purified following application to a preparative silica gel TLC plate; development was carried out with 7:3:2 2-propanol–NH_4OH–H_2O. The band containing pCpA was removed from the plate and triturated with 99:1 CH_3OH–H_2O to provide the desired pCpA. This material was purified further by chromatography on a DEAE-cellulose column; pCpA was isolated as a white powder in 93% yield.

The same procedure was scaled up directly to permit the preparation of larger quantities of pCpA; samples of CpA as large as 40 mg were phosphorylated in excellent yields.

Synthesis of N-Pyroglutamylaminoacyl-pCpA's. Two different methods were developed for the conversion of pCpA to the requisite 2′(3′)-*O*-(*N*-pyroglutamylaminoacyl)pCpA's **6c**. Each of these is illustrated below for a single derivative of **6c**.

Synthesis of **6c** *via an N-(Benzyloxycarbonyl)aminoacyl-pCpA.* *N*-(Benzyloxycarbonyl)-L-phenylglycine (Bachem, Inc.) (14 mg; 0.05 mmol) and 1,1′-carbonyldiimidazole (8 mg; 0.05 mmol) were dissolved in 0.2 mL of dry dimethyl sulfoxide; the solution was stirred over 4A molecular sieves for 30 min under N_2 and then treated with 100 A_{260} units (5 μmol) of pCpA. The reaction mixture was maintained at 25 °C under N_2 for 2 days and was then added dropwise to 6 mL of 1:1:1 *n*-hexane–ether–acetone. The resulting precipitate was collected by centrifugation, dissolved in 100 μL of 0.01 N acetic acid, and applied to a preparative cellulose TLC plate (10 × 20 cm) which was developed with 5:2:3 1-butanol–acetic acid–H_2O. The UV-active band (R_f 0.7–0.8) was removed from the plate and extracted with 10 mL of 0.01 N acetic acid; lyophilization of the extract afforded 22 A_{260} units (22%) of putative bis[*N*-(benzyloxycarbonyl)-L-phenylglycyl]pCpA derivative **5a** (R = C_6H_5). This intermediate was dissolved in 0.01 N acetic acid, and the solution was adjusted to pH 2 with 0.01 N HCl. The solution was stirred at 25 °C for 12 h and then neutralized with Dowex 50W resin (Na^+ form). The solution was then concentrated to a small (~0.1 mL) volume and applied to a preparative cellulose TLC plate (10 × 20 cm). The TLC plate was developed with 5:2:3 1-butanol–acetic acid–H_2O, affording a UV-active band at R_f 0.50–0.55. The material in this band was recovered by extraction with 0.01 N acetic acid. The extract was concentrated to a small volume and lyophilized, affording 17 A_{260} units (77%) of 2′(3′)-*O*-[*N*-(benzyloxycarbonyl)-L-phenylglycyl]-pCpA (**6a**; R = C_6H_5), R_f 0.44 (cellulose TLC, development with 5:2:3 1-butanol–acetic acid–H_2O).

2′(3′)-*O*-[*N*-(Benzyloxycarbonyl)-L-phenylglycyl]pCpA (16 A_{260} units) was dissolved in 0.5 mL of 0.01 N acetic acid containing 50% ethanol and treated with 1 mg of 10% palladium on carbon. The reaction mixture was stirred at 25 °C under H_2 for 12 h. The catalyst was removed by centrifugation, and the supernatant was concentrated to dryness. The residue was dissolved in 0.3 mL of *N*,*N*-dimethylformamide containing 1.0 mg (4.4 μmol) of *N*-(pyroglutamyloxy)-succinimide. The reaction mixture was stirred at 25 °C for 5 h and then added dropwise into 6 mL of acetone. The resulting precipitate was collected by centrifugation and purified by preparative cellulose TLC; development was with 5:2:3 1-butanol–acetic acid–H_2O. The major UV-active band (R_f 0.60–0.65) was removed from the plate and extracted with 0.01 N acetic acid. Concentration of the extract to a small volume and lyophilization afforded 12 A_{260} units (75%) of 2′(3′)-*O*-(*N*-pyroglutamyl-L-phenylglycyl)pCpA (**6c**; R = C_6H_5) as a white solid: λ_{max} (pH 7.0) 258 nm; R_f 0.60 (cellulose TLC, development with 5:2:3 1-butanol–acetic acid–H_2O).

Synthesis of **6c** *via an N-(tert-Butyloxycarbonyl)aminoacyl-pCpA.* *N*-(*tert*-Butyloxycarbonyl)-L-*O*-methyltyrosine (Bachem, Inc.) (15 mg; 0.05 mmol) and 1,1′-carbonyldiimidazole (8 mg; 0.05 mmol) were dissolved in 0.2 mL of dry dimethyl sulfoxide; the solution was stirred over 4A molecular sieves for 30 min under N_2 and then treated with 100 A_{260} units (5 μmol) of pCpA. The reaction mixture was stirred at 25 °C for 48 h and then concentrated and applied to a preparative cellulose TLC plate (10 × 20 cm), which was developed first with acetone and then with 5:2:3 1-butanol–acetic acid–H_2O. The UV-active band was recovered from the plate by extraction with 0.01 N acetic acid; lyophilization afforded 23 A_{260} units of **5b** (R = $CH_3OC_6H_4CH_2$). This compound was dissolved in 0.01 N acetic acid, adjusted to pH 2 with 0.01 N HCl, and stirred at 25 °C for 15 h. The solution was adjusted to pH 4 ($NaHCO_3$), concentrated to 0.5 mL, and applied to a preparative cellulose TLC plate (10 × 20 cm). Development with 5:2:3 1-butanol–acetic acid–H_2O gave a major UV-active band from which 18 A_{260} units (78%) of **6b** (R = $CH_3OC_6H_4CH_2$) was recovered.

2′(3′)-*O*-[*N*-(*tert*-Butyloxycarbonyl)-L-*O*-methyltyrosyl]-pCpA (18 A_{260} units) was treated with 5 mL of trifluoroacetic acid at 25 °C for 3 h. The solution was concentrated, the residue was dissolved in 2 mL of water, and the resulting solution was neutralized with 40% aqueous tetrabutylammonium hydroxide. The solution was concentrated, and the residue was suspended in 0.3 mL of dimethyl sulfoxide and treated with 10 mg (44 μmol) of *N*-(pyroglutamyloxy)-succinimide. The reaction mixture was stirred at 25 °C for 12 h and then applied to a preparative cellulose TLC plate (10 × 20 cm). Development with acetone and then with 5:2:3 1-butanol–acetic acid–H_2O gave a major UV-active band from which 2′(3′)-*O*-(*N*-pyroglutamyl-L-*O*-methyltyrosyl)pCpA (**6c**; R = $CH_3OC_6H_4CH_2$) was isolated as a white solid, yield 14 A_{260} units (78%).

Preparation of tRNA^{Phe}-C_{OH}. A reaction mixture containing 250 μL (total volume) of 0.1 M Tris-HCl, pH 7.5, 3.3 A_{260} units of *E. coli* tRNA^{Phe}, 0.2 M KCl, and 10 mM MgOAc was treated with 0, 0.01, 0.10, 1.00, or 10.0 μg/mL RNase A; the RNase A was added from a stock solution containing 1 mg/mL RNase A in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.1 M NaCl. The digestions were carried out at 0 °C for 1 h.

Individual reaction mixtures were treated with 3 μL of 2-mercaptoethanol (to a final concentration of 170 mM) and then extracted three times with an equal volume of 1:1:0.042 phenol–chloroform–isoamyl alcohol. The aqueous phase was extracted with five portions of ether, and the tRNA was precipitated overnight at −70 °C with 2.5 volumes of cold ethanol. The tRNA was isolated by centrifugation in essentially quantitative yield.

Individual samples of RNase A treated tRNA^{Phe}'s (2.0 A_{260} units) were dissolved in 500 μL (total volume) of Na-Hepes

buffer, pH 6.9, containing 20 mM $MgCl_2$, 30 μM ATP, 3.3 mM dithiothreitol, and 10 mg/mL bovine serum albumin. The incubation mixtures were treated with 90 units of T4 polynucleotide kinase and maintained at 37 °C for 2 h. The reaction mixtures were treated with 20 μL of 4 M NaCl and then with 2.5 volumes of ethanol (12 h, −70 °C). The tRNA was isolated by centrifugation; recovered yields were 80–90%. This material was shown to have the same biochemical properties as $tRNA\text{-}C_{OH}$ prepared by a stepwise procedure (Heckler et al., 1984a).

Analysis of RNase A Treated $tRNA^{Phe}$'s. Reaction mixtures (25-μL total volume) containing 20 mM Tris-HCl, pH 8.7, 10 mM $MgCl_2$, and either 300 μM ATP or 300 μM ATP and 400 μM CTP were treated with 2 μL of yeast CTP-(ATP):tRNA nucleotidyltransferase. The reactions were incubated at 37 °C for 2 h. Ten-microliter aliquots were assayed for amino acid acceptance as described below.

Aminoacylation of the reconstituted tRNA's was carried out in reaction mixtures (53-μL total volume) containing Na-Pipes buffer, pH 7.3, 100 mM KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM ATP, 27 μM [^{3}H]phenylalanine (specific activity 7 Ci/mmol), and 0.06 A_{260} unit of $tRNA^{Phe}$ that had been treated successively with RNase A and T4 polynucleotide kinase. The assays were initiated by the addition of 3 μL of *E. coli* phenylalanyl-tRNA synthetase, maintained at 37 °C for 30 min, and then applied to glass fiber disks that had been presoaked with 25 mM TTAB in 1% acetic acid. The disks were washed thoroughly with 1% acetic acid, dried, and used for determination of radioactivity.

Preparation of N-Pyroglutamylaminoacyl-tRNA Derivatives. Method A. The general procedure is illustrated for the preparation of *N*-pyroglutamyl-L-phenylglycyl-$tRNA^{Phe}$. To 0.8 A_{260} unit of *E. coli* $tRNA^{Phe}\text{-}C_{OH}$ in 70 μL (total volume) of 100 mM Na-Hepes buffer, pH 7.0, containing 30 mM $MgCl_2$, 4 mg/mL bovine serum albumin, 600 μM ATP, and 20% dimethyl sulfoxide was added 0.5 A_{260} unit of *N*-pyroglutamyl-L-phenylglycyl-pCpA and 12 units of T4 RNA ligase. The reaction mixture was maintained at 4 °C for 16 h and then applied to a 1-mL DEAE-cellulose column that had been equilibrated with 50 mM NaOAc buffer, pH 4.5, containing 1 mM $MgCl_2$. The column was washed with the same buffer containing 0.25 M NaCl until a base-line A_{260} value was reached (~12 mL) and then with the same buffer containing 1 M NaCl. The high-salt eluate contained the tRNA and was applied directly to a 1-mL BD-cellulose column that had been equilibrated with 50 mM NaOAc buffer, pH 4.5, containing 1 mM $MgCl_2$ and 1 M NaCl. The column was washed with the same buffer until a base-line A_{260} value was reached (~12 mL) and then with the same buffer containing 25% ethanol. The eluate containing *N*-pyroglutamyl-L-phenylglycyl-$tRNA^{Phe}$ was dialyzed for 2 h against 4 L of 100 μM KOAc containing 1 mM $MgCl_2$ and then concentrated to afford 0.27 A_{260} unit (34%) of *N*-pyroglutamyl-L-phenylglycyl-$tRNA^{Phe}$.

Method B. E. coli N-pyroglutamyl[^{3}H]phenylalanyl-$tRNA^{Phe}$, *N*-pyroglutamyl[^{3}H]valyl-$tRNA^{Val}$, *N*-pyroglutamyl[^{3}H]leucyl-$tRNA^{Leu}$, *N*-pyroglutamyl[^{3}H]tyrosyl-$tRNA^{Tyr}$, and *N*-pyroglutamyl[^{3}H]glycyl-$tRNA^{Gly}$ were prepared by aminoacyl-tRNA synthetase catalyzed activation with the cognate amino acid, followed by acylation with *N*-(pyroglutamyloxy)succinimide. The aminoacylation reactions were carried out in 100 μL (total volume) of Na-Pipes buffer, pH 7.6, containing 1.0 A_{260} unit of purified tRNA isoacceptor, 40 μM ^{3}H-labeled amino acid, 100 mM KCl, 0.5 mM EDTA, 15 mM $MgCl_2$, and 2.5 mM ATP. Reactions were initiated by the addition of 10 μL of unfractionation *E. coli* aminoacyl-tRNA synthetase preparation (Stulberg, 1967) and maintained at 25 °C for 1 h. The incubation mixture was extracted with phenol, and the aqueous phase was extracted with ether and then treated with ethanol to effect precipitation of the tRNA.

Acylation of the [^{3}H]aminoacyl-tRNA's was carried out with 2.5 mg of *N*-(pyroglutamyloxy)succinimide in 200 μL (total volume) of 20 mM triethanolamine, pH 4.6, containing 50% *N*,*N*-dimethylformamide. The reaction mixtures were maintained at 25 °C for 30 min and then adjusted to pH 8.0 with 0.4 N NaOH. After an additional 10 min, the pH of the reaction mixture was lowered to 5.0 by the addition of 1 N HOAc. The tRNA was recovered by ethanol precipitation and then redissolved in 200 μL of 150 mM NaOAc, pH 5.5, and treated with 25 μL of 100 mM $CuSO_4$ to effect hydrolysis of unacylated [^{3}H]aminoacyl-tRNA (Schofield & Zamecnik, 1968). The reaction mixture was maintained at 25 °C for 30 min, and the *N*-pyroglutamyl[^{3}H]aminoacyl-tRNA was then recovered by precipitation with 2.5 volumes of cold ethanol. The tRNA's could also be purified by chromatography on BD-cellulose. Purification of the *N*-pyroglutamyl[^{3}H]-glycyl-$tRNA^{Gly}$ was effected by chromatography on 1-mL BD-cellulose columns at 4 °C; elution was carried out with 50 mM NaOAc, pH 4.5, containing 1 mM $MgCl_2$ and 1 M NaCl until a base-line absorbance at 260 nm was reached (12–15 mL). The *N*-pyroglutamyl[^{3}H]aminoacyl-tRNA's were then recovered by washing with the same buffer containing 25% ethanol. The yields of isolated *N*-pyroglutamyl[^{3}H]aminoacyl-tRNA's resulting from the acylation with *N*-(pyroglutamyloxy)succinimide were 30–60%.

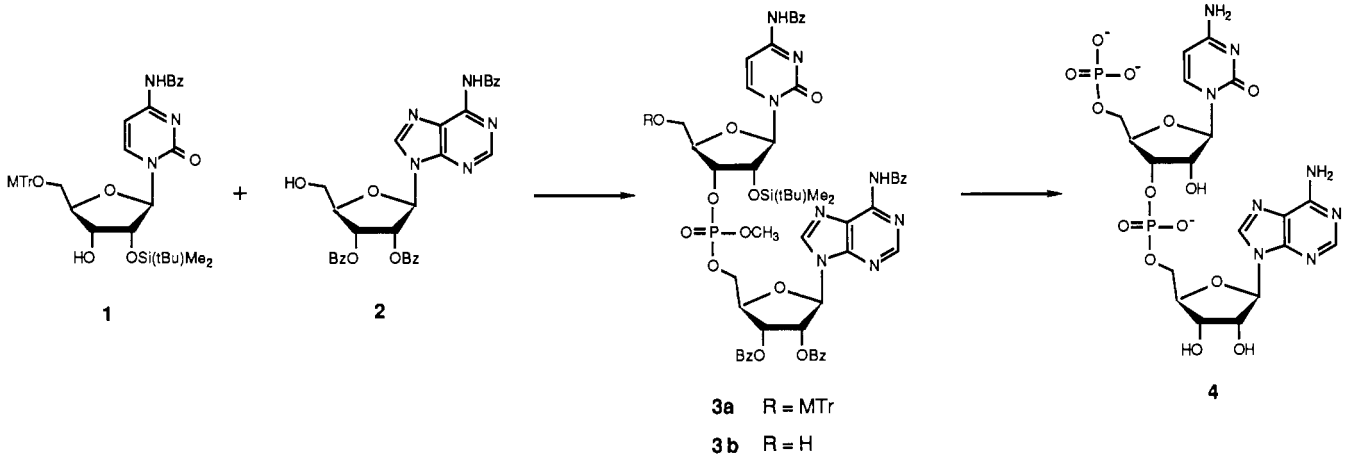
N-Pyroglutamyl[^{3}H]phenylalanyl-$tRNA^{Met}$ and *N*-pyroglutamyl[^{3}H]phenylalanyl-$tRNA^{Val}$ were prepared by the aminoacylation of *E. coli* $tRNA^{Met}$ and $tRNA^{Val}$, respectively, with yeast phenylalanyl-tRNA synthetase (Roe et al., 1973), followed by acylation with *N*-(pyroglutamyloxy)succinimide.

Deblocking and Ribosomal A-Site Function of Chemically Misacylated tRNA's. In a typical experiment, ~0.025 A_{260} unit (40 pmol) of an *N*-pyroglutamylaminoacyl-$tRNA^{Phe}$ in 40 μL (total volume) of 50 mM KOAc, pH 6.0, containing 0.3 unit of pyroglutamate aminopeptidase was incubated at 25 °C for 30 min. A control incubation was carried out in the absence of pyroglutamate aminopeptidase. The derived aminoacyl-$tRNA^{Phe}$ was used directly in a peptidyltransferase reaction, as described below.

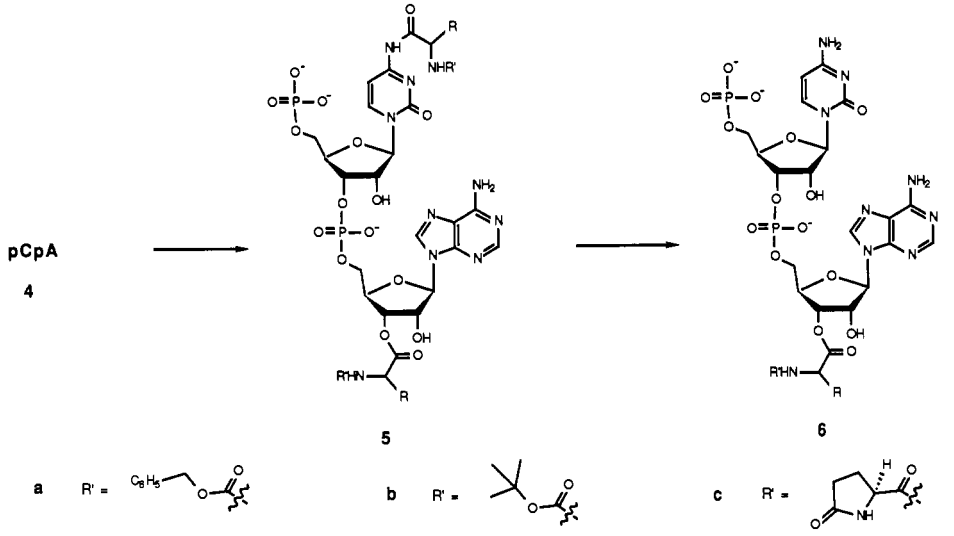
Ribosomal A-site function was determined in 90 μL (total volume) of 100 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 150 mM NH_4Cl, 0.05 mg/mL poly(U), 1 mM 2-mercaptoethanol, and 10 pmol of *N*-acetyl[^{3}H]phenylalanyl-$tRNA^{Phe}$ that had been prebound to 2 A_{260} units of *E. coli* 70S ribosomes for 3 min at 4 °C. The extent of *N*-acetyl[^{3}H]-L-phenylalanyl-$tRNA^{Phe}$ bound to the ribosomes was assayed by filtering an identical reaction mixture, prepared in parallel, through a nitrocellulose filter, which selectively retained the complex. The peptidyltransferase reaction was initiated by addition of the deblocked pyroglutamylaminoacyl-$tRNA^{Phe}$ (vide supra); the reaction was allowed to proceed at 25 °C for 15 min and then terminated by the addition of 90 μL of 1 N NaOH. After 30 min, the reaction mixture was neutralized by the addition of 100 μL of 1.2 N HOAc.

The extent of dipeptide formation was analyzed on a 1-mL BD-cellulose column; the peptidyltransferase reaction mixture was diluted to 1 mL with 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl and 5% ethanol and applied to the column. The column was washed with the same buffer at a flow rate of 0.4 mL/min, and 1-mL fractions were collected and assayed for

Scheme I: Synthetic Pathway for pCpA (4)



Scheme II: Synthetic Routes to *N*-Pyroglutamylaminoacyl-pCpA's (6c)



radioactivity. When base-line radioactivity was reached (~12–15 mL), the formed dipeptide was eluted from the column by washing with 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl, 30% ethanol, and 32% formamide (see arrows in Figures 2 and 3). The radioactivity of these fractions, corrected for the small amount of radioactivity obtained in the absence of any added aminoacyl-tRNA, provided a measure of the extent of dipeptide formation.

Results

As shown in Scheme I, pCpA was prepared by chemical synthesis starting from protected cytidine (**1**) and adenosine (**2**) derivatives. Although the route employed was the same as that used previously (Heckler et al., 1984a,b), the overall yield was increased from ~9% to 33%. Key changes included optimization of the ratio of reactants employed in the initial coupling of **1** and **2** via the agency of methyl phosphorodichloridite and alterations in the work-up procedure for the 2′-*O*-silylated pCpA intermediate. We also developed an alternative method for preparing pCpA by T4 polynucleotide kinase mediated phosphorylation of CpA. Because both T4 polynucleotide kinase and CpA are commercially available, this approach provided pCpA more readily than chemical synthesis. Although significantly more expensive than chemical synthesis of **4**, the enzymatic approach is well suited to the preparation of moderate amounts of pCpA.

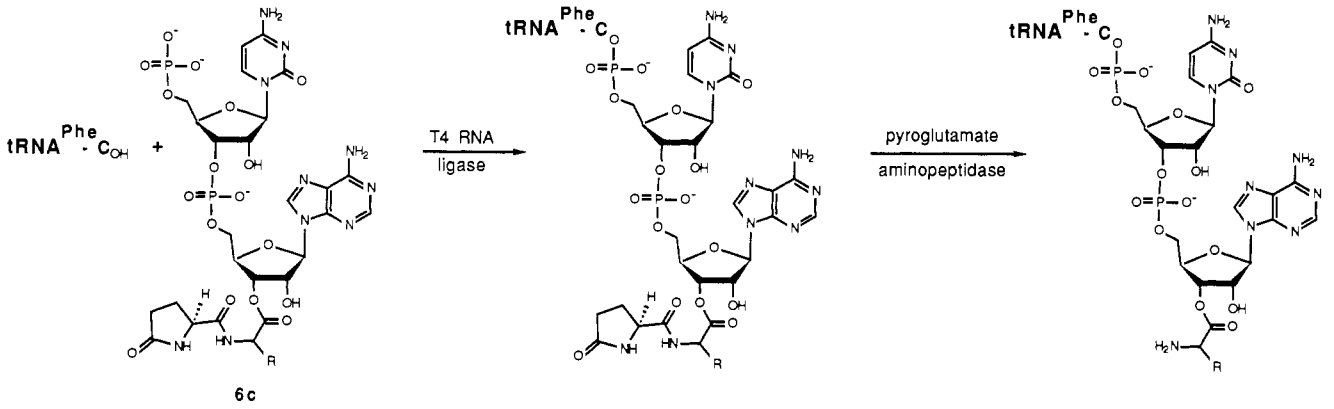
Table I: 2′(3′)-*O*-(*N*-Pyroglutamylaminoacyl)cytidylyl(3′→5′)-adenosine 5′-Phosphates (**6c**)

amino acid	yield (%) **5a(b)**	yield (%) **6a(b)**	yield (%) **6c**	compd **6c** λ_{max} (nm), pH 7	compd **6c** R_f[a]
N-(Benzyloxycarbonyl) Intermediates					
L-phenylalanine	25	40	35	258	0.64
D-phenylalanine	22	38	27	258	0.68
D-tyrosine	33	50	27	258	0.67
L-phenylglycine	22	77	75	258	0.60
D,L-β-phenylalanine	11	56	78	257	0.62
N-(*tert*-Butyloxycarbonyl) Intermediate					
L-*O*-methyltyrosine	23	78	78	258	0.63

[a] Cellulose TLC; development was with 5:2:3 1-butanol–acetic acid–H_2O.

As shown in Scheme II, a few different methods could be employed for the conversion of pCpA (**4**) to the respective *N*-pyroglutamylaminoacyl-pCpA's (**6c**). Most of the examples reported here employed *N*-benzyloxycarbonyl (CBz) derivatives of the individual amino acids. These were used to diacylate pCpA (i.e., **4** → **5a**) via the agency of 1,1′-carbonyldiimidazole; treatment of **5a** with dilute HCl at 25 °C for 12 h effected selective hydrolysis of the N^4-acyl linkage, producing **6a**. Hydrogenolysis of this aminoacylated dinucleotide, followed by acylation with *N*-(pyroglutamyloxy)succinimide, provided **6c**. The yields obtained in the

Scheme III: Preparation and Enzymatic Deblocking of *N*-Pyroglutamylaminoacyl-tRNA^{Phe}'s



individual transformations (**4** → **5a** → **6a** → **6c**) are given for five *N*-pyroglutamylaminoacyl-pCpA's in Table I. A second route to **6c** involved the intermediacy of *N*-(*tert*-butyloxycarbonyl)-(tBoc) protected amino acids, as illustrated for the preparation of the L-*O*-methyltyrosine derivative of pCpA via **5b** and **6b** (Scheme II and Table I). Also investigated successfully was a slightly more direct route to **6c** involving the intermediacy of bis(*N*-pyroglutamylaminoacyl)-pCpA's **5c** (data not shown).

As reported previously (Heckler et al., 1984a), tRNA-C$_{OH}$ has been prepared by controlled digestion of tRNA-CCA$_{OH}$ with venom exonuclease, followed by treatment with yeast CTP(ATP):tRNA nucleotidyltransferase in the presence of CTP, but not ATP. The formed tRNA-CC$_{OH}$ was then treated successively with periodate, lysine, and alkaline phosphatase (Neu & Heppel, 1964). An alternative procedure, which has proved to be simpler operationally, involved digestion of the tRNA with bovine pancreatic ribonuclease A. This converted the tRNA-CCA to tRNA-Cp (Richards & Wyckoff, 1971), the latter of which was dephosphorylated by using the intrinsic 3′-phosphatase activity in T4 polynucleotide kinase (Cameron & Uhlenbeck, 1977). As shown in Figure 1, treatment of tRNA^{Phe} with 0.01 μg/mL ribonuclease A reduced phenylalanine acceptance to 56% of the value obtained for untreated tRNA. Larger amounts of RNase A virtually eliminated phenylalanine acceptor activity, indicating that none of the treated tRNA's had an intact 3′-(CCA) terminus. Consistent with this interpretation, both yeast tRNA^{Phe} and *E. coli* tRNA^{Phe} treated in this fashion with RNase A migrated slightly farther than the respective untreated tRNA's on a denaturing 20% polyacrylamide gel; each treated tRNA^{Phe} produced a single, sharp band on the gel (data not shown). Also shown in the figure was the effect of reconstructing the 3′-terminus of the RNase A digested tRNA^{Phe} following removal of the formed 3′-phosphate with T4 polynucleotide kinase. For example, the tRNA^{Phe} sample treated with 0.1 μg/mL RNase A was aminoacylated 94% as well as a control sample after reconstitution with CTP(ATP):tRNA nucleotidyltransferase and ATP alone, suggesting that the initial RNase A digestion had not removed cytidines from the acceptor stem to any significant extent (i.e., that it was tRNA^{Phe}-CC$_{OH}$). In contrast, treatment with 1 μg/mL RNase A afforded a product whose phenylalanine acceptor activity could be substantially restored by the CTP(ATP):tRNA nucleotidyltransferase only when both CTP and ATP were present. This observation, and the known substrate specificity of RNase A (Richards & Wyckoff, 1971), indicated that this material was the desired tRNA^{Phe}-C$_{OH}$. Of interest from the perspective of a preparatively useful transformation was the observation that even a 10-fold greater concentration of RNase A produced tRNA^{Phe}-C$_{OH}$ that could be reconstituted in the presence of CTP and ATP.

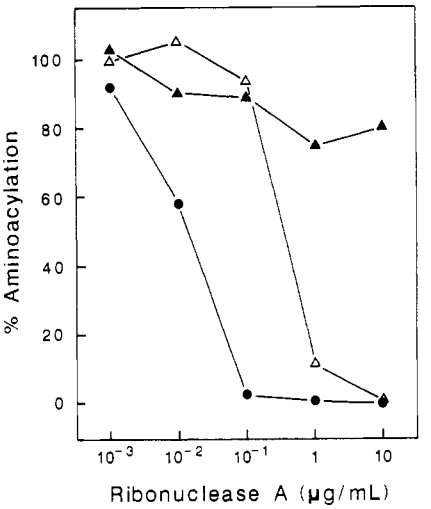


FIGURE 1: Phenylalanine acceptance activity of *E. coli* tRNA^{Phe} that had been treated with various concentrations of RNase A. Samples of the RNase A treated tRNA were aminoacylated with *E. coli* phenylalanyl-tRNA synthetase (●) or else treated with CTP-(ATP):tRNA nucleotidyltransferase in the presence of ATP alone (△) or ATP + CTP (▲) and then aminoacylated. A detailed assay procedure is given under Experimental Procedures.

Table II: Preparation and Ribosomal A-Site Function of Chemically Misacylated tRNA^{Phe}'s[a]

misacylated tRNA	amount of misacylated tRNA produced		amount of dipeptide formed[b]	
	pmol	% yield	pmol	% yield
N-pyroglutamyl-L-phenylalanyl-tRNA^{Phe}	160	40	5.4	100
N-pyroglutamyl-D-phenylalanyl-tRNA^{Phe}	237	20	0	0
N-pyroglutamyl-D-tyrosyl-tRNA^{Phe}	216	22	0	0
N-pyroglutamyl-L-*O*-methyltyrosyl-tRNA^{Phe}	259	40	2.2	70
N-pyroglutamyl-L-phenylglycyl-tRNA^{Phe}	340	35	3.8	70
N-pyroglutamyl-D,L-β-phenylalanyl-tRNA^{Phe}	142	28	0.4	8

[a] See Experimental Procedures for detailed protocols. [b] Yield relative to the amount of dipeptide obtained with authentic *E. coli* phenylalanyl-tRNA^{Phe} under comparable conditions.

N-Pyroglutamylaminoacyl-tRNA's were prepared by T4 RNA ligase mediated coupling of tRNA^{Phe}-C$_{OH}$ and *N*-pyroglutamylaminoacyl-pCpA's (**6c**) (Scheme III); the preparation of six tRNA's in this fashion is summarized in Table

Scheme IV: Preparation of *N*-Pyroglutamylaminoacyl-tRNA's by Acylation of Enzymatically Activated Aminoacyl-tRNA's with *N*-(Pyroglutamyloxy)succinimide

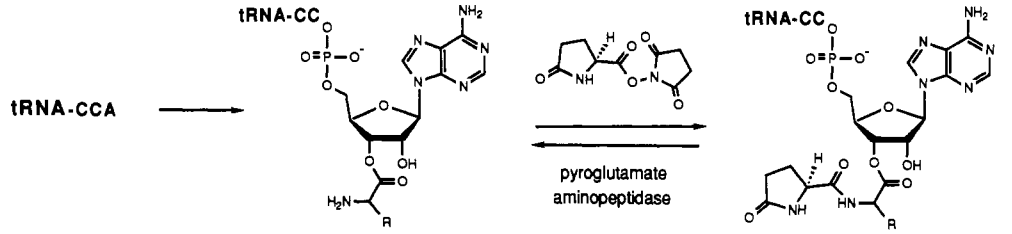


Table III: Deprotection of *N*-Pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} by Pyroglutamate Aminopeptidase[a]

reactants (amounts)	incubation time (min)	Cu^{2+}	amino-acylated tRNA (% of original)
N-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (50 pmol) + pyroglutamate aminopeptidase (0.2 unit)	0	–	100
	5	–	92
	10	+	28
	20	+	30
N-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (50 pmol)	0	–	100
	5	–	83
	10	+	105
	20	+	87

[a] *N*-Pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (50 pmol) was incubated at 25 °C in 100 μL of 100 mM KOAc, pH 6.0, in the presence or absence of 0.2 unit of pyroglutamate aminopeptidase. Aliquots (20 μL) were removed after 0 and 5 min and applied to glass fiber disks that had been presoaked with 25 mM TTAB in 1% acetic acid. Immediately after the 5-min time point, the incubation mixtures were each treated with 0.1 volume of 100 mM $CuSO_4$, and incubation was continued at 25 °C. Additional 20-μL aliquots were removed after 10 and 20 min and applied to glass fiber disks, as indicated above. The dried disks were washed thoroughly with portions of 1% acetic acid, dried, and used for determination of radioactivity.

II. Also prepared to facilitate an assessment of the generality of the enzymatic deblocking with pyroglutamate aminopeptidase were several *N*-pyroglutamyl[^{3}H]aminoacyl-tRNA's accessible by aminoacyl-tRNA synthetase catalyzed activation of a single tRNA isoacceptor, followed by acylation with *N*-(pyroglutamyloxy)succinimide (Scheme IV).

The ability of pyroglutamate aminopeptidase to effect removal of the pyroglutamyl protecting group without concomitant hydrolysis of the aminoacyl linkage in the *N*-pyroglutamylaminoacyl-tRNA's was assayed by the use of Cu^{2+}, which has been shown to catalyze the hydrolysis of the aminoacyl moiety in aminoacyl-tRNA's but not in N-acylated aminoacyl-tRNA's (Schofield & Zamecnik, 1968). Accordingly, when *N*-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (prepared according to Scheme IV) was treated with pyroglutamate aminopeptidase, subsequent precipitation of the tRNA on a TTAB-soaked glass fiber disk also resulted in coprecipitation of the associated [^{3}H]phenylalanine, suggesting that the aminoacyl linkage was still intact (Table III). Subsequent treatment with 10 mM Cu^{2+} effected hydrolysis of the aminoacyl linkage, indicating that the pyroglutamate aminopeptidase had indeed removed the L-pyroglutamate protecting group. Consistent with this interpretation was the observed stability of *N*-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} in the presence and absence of Cu^{2+} when the enzymatic deblocking procedure was omitted (Table III). The ability of pyroglutamate aminopeptidase to effect the deblocking of several additional *N*-pyroglutamyl[^{3}H]aminoacyl-tRNA's was studied with the same techniques; as shown in Table IV several different tRNA's bearing their cognate amino acids were deprotected in this fashion.[2]

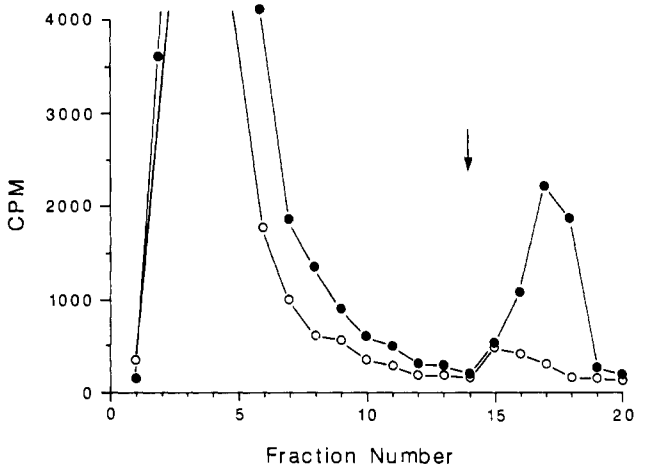


FIGURE 2: Dipeptide formation using *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} and pyroglutamate aminopeptidase treated *N*-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (of severalfold higher specific activity). *N*-Acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} (10 pmol) was prebound to poly(U)-programmed *E. coli* ribosomes. The incubation mixture was treated with 40 pmol of *E. coli* *N*-pyroglutamyl-L-phenylalanyl-tRNA^{Phe} that had been pretreated with 0.2 unit of pyroglutamate aminopeptidase for 10 min at 25 °C. The combined solution was incubated at 25 °C for 15 min and then treated with 1 N NaOH to quench the reaction and effect hydrolysis of peptidyl-tRNA. A parallel reaction was carried out in the same fashion, except that the *N*-pyroglutamyl-L-phenylalanyl-tRNA^{Phe} was not subjected to enzymatic deblocking. Analysis of dipeptide formation was carried out on BD-cellulose columns for the reaction containing (●) and lacking (○) pyroglutamate aminopeptidase. A control was also run in the absence of any aminoacyl-tRNA. Detailed protocols are provided under Experimental Procedures.

That the treatment with pyroglutamyl aminopeptidase actually effected the conversion of pyroglutamylaminoacyl-tRNA's to the respective aminoacyl-tRNA's was demonstrated with *N*-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe}, which had been prepared by the treatment of authentic [^{3}H]-L-phenylalanyl-tRNA^{Phe} with *N*-(pyroglutamyloxy)succinimide followed by separation of the N-acylated product from unreacted phenylalanyl-tRNA^{Phe} on a BD-cellulose column. As shown in Figure 2, when included in a poly(U)-directed ribosomal system containing ~10 pmol of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} prebound to the ribosomal P-site, pyroglutamate aminopeptidase treated *N*-pyroglutamyl-L-phenylalanyl-tRNA^{Phe} acted as an acceptor tRNA, affording ~4.2 pmol of a product that behaved in the same fashion chromatographically as authentic *N*-acetyl-L-phenylalanyl-L-phenyl-

[2] Ostensibly incomplete deprotection was noted in the majority of cases studied, even when high concentrations of pyroglutamate aminopeptidase or Cu^{2+} were employed. That this was an artifact of the assay system, possibly reflecting the insolubilization of free ^{3}H-labeled amino acids on the glass fiber disks by the pyroglutamate aminopeptidase preparation, was suggested both by the apparent *decrease* in deblocking observed as the amount of enzyme preparation was increased and by the efficient utilization of some of the deblocked aminoacyl-tRNA's as acceptors in the peptidyltransferase reaction.

Table IV: Deprotection of *N*-Pyroglutamyl[^{3}H]-L-aminoacyl-tRNA's with Pyroglutamate Aminopeptidase[a]

substrate	TTAB-precipitable [^{3}H]aminoacyl-tRNA (pmol)		% deprotection
	0 min + pyroglutamate aminopeptidase	25 min + pyroglutamate aminopeptidase, Cu^{2+}	
N-pyroglutamyl[^{3}H]-L-valyl-tRNA^{Val}	42.3	25.1	41
N-pyroglutamyl[^{3}H]-L-leucyl-tRNA^{Leu}	13.5	6.7	50
N-pyroglutamyl[^{3}H]-L-tyrosyl-tRNA^{Tyr}	15.8	5.2	67
N-pyroglutamyl[^{3}H]-L-glycyl-tRNA^{Gly}	45.1	34.2	24

[a] The deprotection was carried out for 25 min at 25 °C, as described under Experimental Procedures; the Cu^{2+} treatment was carried out with 0.1 volume of 100 mM $CuSO_4$.

alanine. Absent the initial treatment of *N*-pyroglutamyl-L-phenylalanyl-tRNA^{Phe} with pyroglutamate aminopeptidase, no significant amount of dipeptide was formed (Figure 2).[3]

Also studied was the treatment with pyroglutamate aminopeptidase of *N*-pyroglutamylaminoacyl-tRNA^{Phe}'s prepared by "chemical aminoacylation" (Scheme III) and utilization of the derived aminoacyl-tRNA's as acceptors in the peptidyltransferase reaction. Following purification of the chemically aminoacylated *N*-pyroglutamylaminoacyl-tRNA^{Phe}'s on DEAE-cellulose and BD-cellulose columns, the preparations were each divided into two portions. One of these was treated with pyroglutamate aminopeptidase before the two portions were added to separate incubation mixtures containing *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} prebound to *E. coli* ribosomes plus poly(U). As shown in Table II, chemically aminoacylated L-phenylalanyl-tRNA^{Phe} was as effective in the peptidyltransferase reaction as authentic L-phenylalanyl-tRNA^{Phe} (i.e., as a sample prepared by admixture of L-phenylalanine and tRNA^{Phe} in the presence of phenylalanyl-tRNA synthetase). No dipeptide was formed in the incubation mixture containing *N*-pyroglutamyl-L-phenylalanyl-tRNA^{Phe} that had not been pretreated with pyroglutamate aminopeptidase. Although neither chemically aminoacylated *N*-pyroglutamyl-D-phenylalanyl-tRNA^{Phe} nor *N*-pyroglutamyl-D-tyrosyl-tRNA^{Phe} participated in dipeptide formation to a detectable extent following treatment with pyroglutamate aminopeptidase, the enzyme-treated L-*O*-methyltyrosyl-tRNA^{Phe} and L-phenylglycyl-tRNA^{Phe} were reasonably efficient acceptors in the peptidyltransferase reaction (Table II and Figure 3). Also shown in the table are the results obtained when *N*-pyroglutamyl-D,L-β-phenylalanyl-tRNA^{Phe} was employed as a potential acceptor in the peptidyltransferase reaction following treatment with pyroglutamate aminopeptidase. As indicated, 0.4 pmol (8%) of putative *N*-acetyl-L-phenylalanyl-D,L-β-phenylalanine was obtained; the same observation was made reproducibly in independent experiments. Control experiments which employed *N*-pyroglutamyl-D,L-β-phenylalanyl-tRNA^{Phe} that had not been treated with pyroglutamate aminopeptidase, or which omitted aminoacyl-tRNA^{Phe}, afforded no detectable dipeptide.

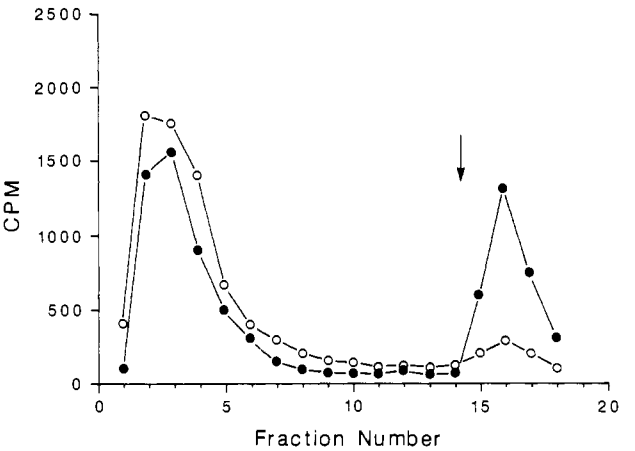


FIGURE 3: Dipeptide formation using *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} and pyroglutamate aminopeptidase treated *N*-pyroglutamyl-L-phenylglycyl-tRNA^{Phe}. Dipeptide formation and analysis were carried out as described in the legend to Figure 2, for reactions containing (●) and lacking (○) pyroglutamate aminopeptidase.

Discussion

In previous reports (Heckler et al., 1983, 1984a,b, 1988), we have described the preparation of N-acylated aminoacyl-tRNA's via the T4 RNA ligase mediated condensation of tRNA-C_{OH} and N-acylated pCpA's. The derived tRNA's, many of which contained noncognate or non naturally occurring amino acids, were studied as donors in the peptidyltransferase reaction. These studies demonstrated that structurally diverse peptidyl-tRNA analogues functioned well as donors; products formed with reasonable efficiency in this fashion included *N*-acetyl-L-β-phenylalanyl-L-phenylalanyl-tRNA^{Phe}, *N*-acetyl-D-β-phenylalanyl-L-phenylalanyl-tRNA^{Phe}, *N*-acetyl-L-tyrosyl-L-phenylalanyl-tRNA^{Phe}, *N*-acetylphenylglycyl-L-phenylalanyl-tRNA^{Phe}, *trans*-cinnamyl-L-phenylalanyl-tRNA^{Phe}, and 3-phenylpropionyl-L-phenylalanyl-tRNA^{Phe} (Heckler et al., 1983, 1988). In one case, by the use of a peptidyl-tRNA analogue containing two electrophilic sites, it was possible to demonstrate the formation of a dipeptide analogue of altered connectivity (Roesser et al., 1986). In the aggregate, these studies suggest that the ribosome is capable of mediating transformations significantly broader in scope than those observed with the 20 amino acids that ordinarily participate in peptide bond formation.

To facilitate a better understanding of structural requirements for participation as an acceptor in the peptidyltransferase reaction, we have developed an approach for the preparation of aminoacyl-tRNA analogues bearing noncognate aminoacyl moieties. This involved the T4 RNA ligase mediated condensation of tRNA-C_{OH} with an aminoacyl-pCpA derivative, the latter of which contained a protecting group to preclude hydrolysis of the chemically labile aminoacyl linkage during the ligation reaction or subsequent purification.

The choice of a protecting group for the aminoacyl moiety was constrained by the requirement that this group be removable under conditions compatible with maintainence of the activated ester linkage between the amino acid and tRNA and with the integrity of the tRNA molecule itself. Accordingly, we tested several commercially available enzyme preparations for their abilities to effect deblocking of suitably N-acylated aminoacyl-tRNA substrates under conditions that did not also result in deesterification of the tRNA. The course of deblocking and deesterification was monitored by the development of sensitivity to deacylation by aqueous Cu^{2+}, a

[3] In control experiments, admixture of authentic L-phenylalanyl-tRNA^{Phe} to a poly(U)-directed protein-synthesizing system containing 10 pmol of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} resulted in the formation of ~4.3 pmol of putative *N*-acetyl-L-phenylalanyl-L-phenylalanine.

property known to be characteristic of aminoacyl-tRNA's having free α-NH_2 groups (Schofield & Zamecnik, 1968) (cf. Tables III and IV). Treatment of *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} with acylase I effected deesterification of the substrate; alteration of incubation conditions gave deesterification to varying extents, but none of the product mixtures exhibited enhanced sensitivity to deacylation by Cu^{2+} (supplementary material table). Likewise, treatment of L-phenylalanyl-L-phenylalanyl-tRNA^{Phe} with porcine pepsin A gave deesterification but no L-phenylalanyl-tRNA^{Phe}. Also tested was proline-specific endopeptidase (Yoshimoto et al., 1978). Incubation of this enzyme with glycyl-L-prolyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} gave some deesterification, and possibly a small amount of [^{3}H]-L-phenylalanyl-tRNA^{Phe} (supplementary material table). In contrast, while treatment of *N*-pyroglutamyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} with pyroglutamate aminopeptidase resulted in only 7% deesterification, subsequent treatment with Cu^{2+} diminished tRNA-associated radioactivity by 76%, suggesting that the enzyme had indeed deacylated the substrate, producing [^{3}H]-L-phenylalanyl-tRNA^{Phe} (supplementary material table). This observation was verified in subsequent experiments, including some that measured deacylation as a function of time (Table III). On the basis of these results, pyroglutamate aminopeptidase was studied further.

Four purified *E. coli* tRNA isoacceptors were esterified with their cognate amino acids by the use of the corresponding aminoacyl-tRNA synthetases; each of these was then N-acylated on the aminoacyl moiety with *N*-(pyroglutamyloxy)succinimide. As indicated in Table IV, each of the four *N*-pyroglutamyl[^{3}H]-L-aminoacyl-tRNA's could be deblocked with pyroglutamate aminopeptidase.[2] In addition, purified samples of *E. coli* tRNA^{Met} and *E. coli* tRNA^{Val} were misacylated enzymatically with yeast phenylalanyl-tRNA synthetase (Roe et al., 1973) and converted to the respective *N*-pyroglutamyl derivatives. Treatment of these misacylated substrates with pyroglutamate aminopeptidase gave phenylalanyl-tRNA's to the extent of ~45% and 50, respectively, as judged by Cu^{2+} assay (vide supra).[2] That the deblocking procedure actually afforded free aminoacyl-tRNA's was established for deblocked *E. coli* [^{3}H]-L-phenylalanyl-tRNA^{Phe}, which was shown to form dipeptide to the same extent as authentic L-phenylalanyl-tRNA^{Phe} when used as an acceptor in the peptidyltransferase reaction (Figure 2).

Also treated with pyroglutamate aminopeptidase were six different misacylated *E. coli* tRNA^{Phe}'s prepared by T4 RNA ligase mediated chemical aminoacylation (Heckler et al., 1983, 1984a,b, 1988). Putative deblocked L-phenylalanyl-tRNA^{Phe} prepared by this procedure participated as an acceptor in the peptidyltransferase reaction fully as well as the authentic aminoacyl-tRNA. Two tRNA^{Phe}'s bearing noncognate amino acids, i.e., *N*-pyroglutamyl-L-*O*-methyltyrosyl-tRNA^{Phe} and *N*-pyroglutamyl-L-phenylglycyl-tRNA^{Phe}, were also able to participate as acceptors in the peptidyltransferase reaction following enzymatic deblocking (Table II; Figure 3). This observation verifies both the ability of pyroglutamate aminopeptidase to utilize these species as substrates and the ability of the formed aminoacyl-tRNA's to participate in peptide bond formation. In contrast, neither *N*-pyroglutamyl-D-phenylalanyl-tRNA^{Phe} nor *N*-pyroglutamyl-D-tyrosyl-tRNA^{Phe} acted as an acceptor in the peptidyltransferase reaction following treatment with pyroglutamate aminopeptidase, and *N*-pyroglutamyl-D,L-β-phenylalanyl-tRNA^{Phe} produced dipeptide only to the extent of 8%. The finding that the putative D-aminoacyl-tRNA^{Phe}'s failed to act as acceptors in the peptidyltransferase reaction parallels observations made previously for studies of ribosomal A-site and P-site function (Yamane et al., 1981; Heckler et al., 1983, 1988), although this may not preclude the participation of such aminoacyl-tRNA's in appropriately constituted in vitro protein biosynthesizing systems (Calendar & Berg, 1967).

The results obtained with *N*-pyroglutamyl-D,L-β-phenylalanyl-tRNA^{Phe} are particularly interesting from a mechanistic perspective in spite of the low yields obtained since they indicate that the aminoacyl-tRNA in the ribosomal A-site need not have the nucleophile attached to C^{α} of the aminoacyl moiety to permit participation in peptide bond formation. At present, it is not clear whether the yield of dipeptide reflects an intrinsic property of aminoacyl-tRNA's bearing β-amino acids or is also limited by factors such as inefficient deacylation of the *N*-pyroglutamyl protecting group by pyroglutamate aminopeptidase or binding of the potentially unproductive D isomer to the ribosome (Heckler et al., 1988). Assuming that peptide bond formation by β-amino acids is intrinsically less favorable than that for α-amino acids, it will be particularly interesting to determine whether the facility of peptide bond formation by β-aminoacyl-tRNA's can be influenced by simple alteration of experimental parameters.

Pyroglutamate aminopeptidase has been isolated as a soluble enzyme from brain and hypothalamic tissue (Bauer & Kleinkauf, 1980; Browne & O'Cuinn, 1983) and also from other animal tissues (Szewczuk & Kwiatkowska, 1970; Mudge & Fellow, 1973; Armentrout, 1969) and microorganisms (Armentrout, 1969; Tsuru et al., 1978; Fujiwara et al., 1979, 1981). The enzymes from these sources all have broad substrate specificity, including the ability to degrade certain peptide hormones such as thyrotropin-releasing hormone (Prasad & Peterkovsky, 1976; Prasad et al., 1977; Taylor & Dixon, 1978). Almost all of the *N*-L-pyroglutamyl L-amino acids tested as substrates for the several enzyme preparations have been hydrolyzed at some reasonable rate [Armentrout, 1969; Armentrout & Doolittle, 1961; Uliana & Doolittle, 1969; Orlowski & Meister, 1971; Delange & Smith, 1971; Doolittle (1972) and references cited therein]; the single exception was *N*-pyroglutamyl-L-proline, which was reported not to be a substrate for the *Pseudomonas fluorescens* enzyme (Uliana & Doolittle, 1969). In addition, *P. fluorescens* pyroglutamate aminopeptidase has been reported to hydrolyze *N*-pyroglutamyl-D-alanine, albeit slowly (Uliana & Doolittle, 1969), and the *Bacillus subtilis* enzyme used *N*-pyroglutamyl-β-naphthylamide and *N*-pyroglutamylanilide as substrates with reasonable efficiency (Szewczuk & Mulczyk, 1969). In the present study, efforts to devise a useful assay to monitor release of L-pyroglutamic acid from *N*-pyroglutamylaminoacyl-tRNA's, or to devise a simpler substrate reasonably predictive of the behavior of such tRNA's as substrates for calf liver glutamate aminopeptidase, were frustrated by technical difficulties; the direct assay of N-deacylation of *N*-pyroglutamylaminoacyl-tRNA's continues to be an important technical goal.

In spite of the technical uncertainty in the enzymatic deblocking step noted above, the present results extend ongoing studies of aminoacyl-tRNA function in peptide bond formation in a few important ways. At a methodological level, these include more facile access to aminoacyl-pCpA's and tRNA-C_{OH}, as well as the first examples of preparation of misacylated aminoacyl-tRNA's via chemical aminoacylation [see, however, Baldini et al. (1988)]. Key features of the pyroglutamyl protecting group included successful stabilization of the aminoacyl moiety of *N*-pyroglutamylaminoacyl-pCpA's during

their ligation to tRNA-C_{OH} and subsequent chromatographic purification of the derived *N*-pyroglutamylaminoacyl-tRNA's, compatibility with the use of T4 RNA ligase that permitted the preparation of *N*-pyroglutamylaminoacyl-tRNA's in acceptable yields, and the ability of most or all of the formed *N*-pyroglutamylaminoacyl-tRNA's to act as substrates for calf liver pyroglutamate aminopeptidase without concomitant deesterification. The successful separation of *N*-pyroglutamyl[^{3}H]-L-glycyl-tRNA^{Gly} on BD-cellulose (data not shown) represents the first example of purification of a nonaromatic chemically aminoacylated tRNA on this support and presumably reflects a favorable physicochemical property of the pyroglutamyl group. Assuming that this behavior obtains for chemically aminoacylated tRNA's bearing other nonaromatic amino acids, it would greatly extend the variety of purified aminoacyl-tRNA's accessible for study.

The potential of these newly accessible aminoacyl-tRNA analogues to extend our understanding of peptidyltransferase and provide access to polypeptides of altered structure is illustrated clearly by the behavior of L-*O*-methyltyrosyl-tRNA^{Phe} and L-phenylglycyl-tRNA^{Phe}, both of which bound to the A-site of *E. coli* ribosomes programmed with poly(uridylic acid) and participated in peptide bond formation. Given their efficiency in this process (Table II) and the known lack of specificity for P-site function (Heckler et al., 1983, 1984a,b, 1988), it seems reasonable to anticipate that these species could also be incorporated into proteins in response to a natural mRNA. The introduction of single, misacylated tRNA isoacceptors into a protein biosynthesizing system would logically result in the introduction of a structurally altered amino acid into one or more (predetermined) sites in the derived proteins.

The present study clearly indicated that the formation of a dipeptide of altered connectivity was accessible by the use of a tRNA activated with a β-amino acid. This finding complements our earlier observation for the peptidyl-tRNA analogue *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe}, which also participated in the formation of a peptide analogue of altered connectivity (Roesser et al., 1986). Although subject to the ambiguities of interpretation noted above, the lower yield of dipeptide observed in the present case could reflect a greater structural stringency for ribosomal function of aminoacyl-tRNA's. More precise definition of the ability of aminoacyl-tRNA's of altered structure to participate in the peptidyltransferase reaction should now be possible.

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Supplementary Material Available

Table presenting data on the attempted enzymatic deprotection of N-acylated L-phenylalanyl-tRNA^{Phe}'s (1 page). Ordering information is given on any current masthead page.

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Human and Rat Malignant-Tumor-Associated mRNAs Encode Stromelysin-like Metalloproteinases†

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ABSTRACT: Rat transin and human stromelysin 2 mRNAs, which have been associated with malignant tumors, code for potential proteins with significant sequence homology to the metalloproteinases collagenase and stromelysin. We have used an expression system that allows easy purification of these proteins after transfection of COS cells with a vector containing the corresponding cDNA. This system has allowed us to prepare transin and stromelysin 2 as active proteinases that are inhibited by inhibitors of metalloproteinases. Further analysis of these enzymes indicates that they degrade several components of the extracellular matrix including collagen types III, IV, and V and fibronectin, as well as gelatins formed from several denatured collagen types. In addition, both transin and stromelysin 2 are capable of activating procollagenase in vitro. Thus, in malignant tumors these proteinases may act, both directly and indirectly, to degrade the extracellular matrix and permit tumor invasion of neighboring tissues.

The extracellular matrix (ECM)[1] plays many important roles in the establishment and structural integrity of tissues. Although turnover of the ECM components is normally very low in the adult, an increase in this rate is a necessary event during many normal biological processes including cell migration during development, wound healing, angiogenesis, or postpartum uterine involution. Uncontrolled ECM degradation can have severe consequences, however, as in pathological conditions such as rheumatoid arthritis or tumor invasion and metastasis [for reviews see Mullins and Rohrlich (1983), Yamada (1983), Murphy and Reynolds (1985), and Tryggvason et al. (1987)].

The degradation of the ECM is accomplished through the action of several proteolytic enzymes, including members of the metalloproteinase family (Tryggvason et al., 1987). Several members of this metalloproteinase family have been characterized, including the following: collagenase, which

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[1] Abbreviations: ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; TIMP, tissue inhibitor of metalloproteinases; SV40, simian virus 40; IgG, immunoglobulin G; APMA, (4-aminophenyl)mercuric acetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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