Construction of Modified Ribosomes for Incorporation of D-Amino Acids into Proteins†

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Received May 17, 2006; Revised Manuscript Received September 28, 2006

ABSTRACT: While numerous biologically active peptides contain D-amino acids, the elaboration of such species is not carried out by ribosomal synthesis. In fact, the bacterial ribosome discriminates strongly against the incorporation of D-amino acids from D-aminoacyl-tRNAs. To permit the incorporation of D-amino acids into proteins using *in vitro* protein-synthesizing systems, a strategy has been developed to prepare modified ribosomes containing alterations within the peptidyltransferase center and helix 89 of 23S rRNA. S-30 preparations derived from colonies shown to contain ribosomes with altered 23S rRNAs were found to exhibit enhanced tolerance for D-amino acids and to permit the elaboration of proteins containing D-amino acids at predetermined sites. Five specific amino acids in *Escherichia coli* dihydrofolate reductase and *Photinus pyralis* luciferase were replaced with D-phenylalanine and D-methionine, and the specific activities of the resulting enzymes were determined.

D-Amino acids are widely distributed in living organisms, and they are utilized in a number of contexts. However, only L-amino acids are substrates for ribosomal protein synthesis. All published data concerning the ribosomal incorporation of D-amino acids into proteins *in vivo* and *in vitro* have indicated a strong bias against their incorporation (*1*−*6*); D-amino acid containing peptides and proteins result from post-translational modification (*7*, *8*) or nonribosomal synthesis (*9*). Discrimination between L and D isomers of amino acids has been shown at all stages of translation, including tRNA activation (*10*, *11*).

Nonetheless, the production of D-aminoacyl-tRNA by some aminoacyl-tRNA synthetases has been established *in vitro*, and the toxicity of some D-amino acids is associated with their accumulation in cells (*10*). Likewise, while there is limited data containing specificity of the ribosome for L-amino acids (*12*−*15*), the available data have demonstrated that the specificity for L-amino acids is not absolute. D-Amino acids can bind both in the peptidyltransferase acceptor and donor (A and P) sites of the ribosome and compete with the L isomers (*12*, *13*). Dipeptide synthesis *in vitro* has shown that amino acids such as D-Phe and D-Tyr can participate in peptide bond formation but much less efficiently than the L isomers (*14*, *15*). However, attempts to incorporate D-amino acids into proteins using chemically misacylated suppressor tRNAs in cell-free protein-synthesizing systems have been unsuccessful (*2*, *16*).

The selectivity for L-amino acid participation in peptide bond formation is ultimately defined by the peptidyltransferase center (PTC)[1] of the ribosome, which is constituted by 23S rRNA and ribosomal proteins. Logically, alteration of the PTC should make it possible to facilitate the incorporation of D-amino acids into proteins, at least in a cell-free system. In recent years, the technique of artificial ribosome preparation has been developed and used widely for studying different aspects of ribosome action and the mechanism of drug resistance (*17*−*23*). Clearly, a key issue in ribosome engineering is changing ribosome specificity without significantly decreasing ribosome activity and fidelity. It has been well-demonstrated that peptide bond formation takes place in a highly conserved region of the largest rRNA (central part of domain V of 23S rRNA in *Escherichia coli*) (*24*, *25*) and is central to the efficiency and accuracy of translation.

While mutations in these positions can affect cell survival (*26*−*28*), it has been demonstrated recently that substitutions in this region of rRNA can still afford functional ribosomes, albeit those that form peptide bonds *in vitro* at a diminished rate (*29*−*31*). Critically, such ribosomal mutants tend to be less accurate, arguing for their potential utility in incorporating D-amino acids. In a recent preliminary account, we described the purposeful mutation of the PTC and the selection of ribosomal mutants capable of enhanced incorporation of D-amino acids (*32*). Presently, we provide a detailed description of experiments that afforded modified ribosomes capable of incorporating D-amino acids.

EXPERIMENTAL PROCEDURES

General Methods and Materials. Tris, acrylamide, bisacrylamide, urea, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), dihydrofolic acid, glycerol,

† This study was supported by the National Institutes of Health Research Grant CA77359, awarded by the National Cancer Institute.

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[1] Abbreviations: DHFR, dihydrofolate reductase; PTC, peptidyltransferase center; NVOC, nitroveratryloxycarbonyl; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; LRMS, low-resolution mass spectrum; ESI, electrospray ionization; DMF, *N*,*N*-dimethylformamide; LB, Luria−Bertani broth; IPTG, isopropyl-*β*-D-thiogalactopyranose; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBE, Tris−boric acid−EDTA; PCR, polymerase chain reaction; BSA, bovine serum albumin; MES, 4-morpholinemethanesulfonate.

10.1021/bi060986a CCC: $33.50 © 2006 American Chemical Society
Published on Web 12/01/2006

ampicillin, thiostrepton, kanamycin, chloramphenicol, dNTPs, and ddTTP were purchased from Sigma (St. Louis, MO). ^{35}S-Methionine (10 μCi/μL), [γ-^{32}P]ATP (10 μCi/μL), ^{3}H-amino acid mixture (Leu, Lys, Phe, Pro, and Tyr) (10 μCi/μL), and BrCN were obtained from Amersham (Pitscataway, NJ). Amino acid mixture, pAltex-Ex2 plasmid, BL-21 (DE-3) and JM-109 competent cells (transformation efficiency $> 10^8$), T4 RNA ligase, T4 polynucleotide kinase, endonuclease *Hin*dIII from *Haemophilus influenzae*, *Not*I from *Nocardia otitdis*, and *Nco*I from *Nocardia corallina* were from Promega (Madison, WI). Taq DNA ligase, endonucleases *Dpn*I from *Diplococcus pneumoniae*, *Bam*HI from *Bacillus amyliquefaciens H*, *Kpn*I from *Klebsiella pneumoniae OK*8, and *Eco*RI from *E. coli RY* 13 were obtained from New England Biolabs (Beverly, MA). Pfu DNA polymerase as well as pET28c and pUC18 vectors were purchased from Novagene (Madison, WA). Plasmid pNot was kindly provided by Dr. Knud H. Nierhaus (Max-Plank-Institute for Molecular Genetics, Berlin, Germany). Synthetic oligonucleotides were obtained from Invitrogen Life Technologies (Rockville, MD).

"QiagenPlasmid Midi" (Qiagen, Inc., Valencia, CA) and "Sequenase version 2.0 T7 DNA polymerase" (GE Healthcare Biosciences, Piscataway, NJ) kits were used for plasmid purification and sequencing. $tRNA^{Phe}_{CUA}$ was prepared using plasmid pYRNA8, encoding modified yeast $tRNA^{Phe}_{CUA}$ (lacking the 3′-terminal pCpA) by use of an AmpliScribe T7 RNA polymerase transcription kit (Epicentre Technologies, Madison, WI) as described (*33*). An "Altered Sites II" kit (Promega, Madison, WI) was used for site-directed mutagenesis of pAltex-Ex2.

Phosphorimager analysis was performed using a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 3.2 software. Radioactivity measurements were made using a Beckman LS-100C liquid scintillation counter. Fluorescence spectral measurements were made using a Hitachi F2000 fluorescence spectrophotometer. Ultraviolet and visual spectral measurements were made using a Perkin-Elmer Lambda 20 spectrophotometer.

*N-6-Nitroveratryloxycarbonyl-D-methionine (**1**).* To a solution of 184 mg (1.23 mmol) of D-methionine in 175 mL of 67 mM potassium phosphate buffer at pH 7.2 was added 23.5 mg (12 units) of L-amino acid oxidase. The reaction mixture was incubated at 37 °C for 4 h in an open flask with periodic shaking. The volume was reduced to about 20 mL under diminished pressure followed by the addition of 262 mg (2.47 mmol) of Na_2CO_3 and 342 mg (1.24 mmol) of nitroveratryloxycarbonyl (NVOC) chloride in 10 mL of dioxane. The reaction mixture was stirred at 25 °C for 12 h, acidified to pH 3.0, and then extracted with three 5 mL portions of CH_2Cl_2. The combined organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 2 cm). Elution with a gradient of methanol in dichloromethane (2 → 15%) gave compound **1** as a yellow solid: yield, 198 mg (43%); ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$) δ, 2.08 (s, 3H), 2.10 (m, 2H), 2.54 (m, 2H), 3.92 (s, 3H), 3.96 (s, 3H), 4.46 (m, 1H), 5.45 (s, 2H), 5.80 (m, 1H), 6.98 (s, 1H), and 7.63 (s, 1H); mass spectrum (FAB), *m*/*z* 388.0944 $(M)^+$ ($C_{15}H_{20}N_2O_8S$ requires 388.0940).

*N-6-Nitroveratryloxycarbonyl-D-methionine Cyanomethyl Ester (**2**).* To a solution containing 35 mg (0.09 mmol) of *N*-NVOC-D-methionine (**1**) in 1.8 mL of dry acetonitrile was added 63 μL (0.45 mmol) of triethylamine followed by 29 μL (0.45 mmol) of chloroacetonitrile under argon at 25 °C. The reaction mixture was stirred at 25 °C for 18 h. The solvent was concentrated under diminished pressure. The residue was dissolved in 7 mL of dichloromethane and acidified to pH 2.0 with 0.5 M $NaHSO_4$. The mixture was extracted with three 4 mL portions of CH_2Cl_2. The combined organic extract was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1 cm). Elution with ethyl acetate−hexane afforded *N*-NVOC-D-methionine cyanomethyl ester (**2**) as a yellow foam: yield, 24 mg (62%); ^{1}H NMR (acetone-d_6) δ, 2.14 (s, 3H), 2.21 (m, 2H), 2.72 (m, 2H), 4.00 (s, 3H), 4.02 (s, 3H), 4.60 (m, 1H), 5.08 (s, 2H), 5.52 (q, 2H, J = 15 Hz), 7.24 (s, 1H), and 7.77 (s, 1H); mass spectrum (FAB), *m*/*z* 427.1038 $(M)^+$ ($C_{17}H_{21}N_3O_8S$ requires 427.1049).

*N-6-Nitroveratryloxycarbonyl-D-methionine pdCpA Ester (**3**).* To a conical vial containing 7.0 mg (16.5 μmol) of *N*-NVOC-D-methionine cyanomethyl ester (**2**) was added a solution of 4.5 mg (3.3 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 50 μL of freshly distilled *N*,*N*-dimethylformamide (DMF). The reaction mixture was stirred at room temperature for 3 h. A 2 μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN/50 mM NH_4-OAc at pH 4.5 and was analyzed by high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc at pH 4.5 over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN/50 mM NH_4OAc at pH 4.5 and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **3** (retention time of 25−26 min) was recovered from the appropriate fractions as a colorless solid by lyophilization: yield, 1.2 mg (36%); low-resolution mass spectrum (LRMS) electrospray ionization (ESI), *m*/*z* 1007.2 $(M + H)^+$; theoretical, *m*/*z* 1007.2.

*N-6-Nitroveratryloxycarbonyl-D-phenylalanine (**4**).* To a solution containing 204 mg (1.23 mmol) of D-phenylalanine in 175 mL of 67 mM potassium phosphate buffer at pH 7.2 was added 23.5 mg (12 units) of L-amino acid oxidase. The reaction mixture was incubated at 37 °C for 4 h in an open flask with periodic shaking. The volume was reduced to about 20 mL under diminished pressure followed by the addition of 262 mg (2.47 mmol) of Na_2CO_3 and 342 mg (1.24 mmol) of NVOC chloride in 10 mL of dioxane. The reaction mixture was stirred at 25 °C for 18 h, acidified to pH 3.0, and then extracted with three 5 mL portion of CH_2Cl_2. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (22 × 3 cm). Elution with 10% methanol in dichloromethane gave compound **4** as a light yellow solid: yield, 286 mg (57%); ^{1}H NMR (acetone-d_6) δ, 3.08 (dd, 1H, J = 14.0, 9.4 Hz), 3.32 (dd, 1H, J = 14.1, 4.8 Hz), 3.92 (s, 3H), 3.97 (s, 3H), 4.57 (m, 1H), 5.46 (q, 2H, J = 15.5 Hz), 6.91 (d, 1H, J = 8.1 Hz), 7.16 (s, 1H), 7.27−7.36 (m, 5H), and 7.74 (s, 1H); mass spectrum (FAB), *m*/*z* 404.1213 $(M)^+$ ($C_{19}H_{20}N_2O_8$ requires 404.1220).

*N-6-Nitroveratryloxycarbonyl-D-phenylalanine Cyanomethyl Ester (***5***).* To a solution containing 60 mg (0.15 mmol) of *N*-NVOC-D-phenylalanine (**4**) in 3 mL of dry acetonitrile was added 105 μL (0.75 mmol) of triethylamine followed by 48 μL (0.75 mmol) of chloroacetonitrile under argon at 25 °C. The reaction mixture was stirred at 25 °C for 24 h. The solvent was concentrated under diminished pressure. The residue was dissolved in 6 mL of dichloromethane and acidified to pH 2.0 with 0.5 M $NaHSO_4$. The mixture was extracted with three 10 mL portions of CH_2Cl_2. The combined organic extract was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1 cm). Elution with 1:1 ethyl acetate/hexane afforded *N*-NVOC-D-phenylalanine cyanomethyl ester (**5**) as a yellow solid: yield, 48 mg (73%); ^{1}H NMR (acetone-d_6) δ, 3.13 (dd, 1H, J = 14.1, 9.3 Hz), 3.30 (dd, 1H, J = 14.0, 5.2 Hz) 3.94 (s, 3H), 3.98 (s, 3H), 4.67 (m, 1H), 5.06 (s, 2H), 5.46 (q, 2H, J = 15.3 Hz), 6.91 (d, 1H, J = 18.1 Hz), 7.19 (d, 2H, J = 7.8 Hz), 7.29−7.37 (m, 4H), and 7.75 (s, 1H); mass spectrum (FAB), *m*/*z* 443.1330 $(M)^+$ ($C_{21}H_{21}N_3O_8$ requires 443.1328).

*N-6-Nitroveratryloxycarbonyl-D-phenylalanine pdCpA Ester (***6***).* To a conical vial containing 2.9 mg (6.62 μmol) of *N*-6-NVOC-D-phenylalanine cyanomethyl ester (**5**) was added a solution of 1.8 mg (1.32 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 20 μL of freshly distilled DMF. The reaction mixture was stirred at room temperature for 3.5 h. A 2 μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN/50 mM NH_4OAc at pH 4.5 and was analyzed by HPLC on a C_{18} reversed-phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc at pH 4.5 over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN/50 mM NH_4OAc at pH 4.5 and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **6** (retention time of 28 min) was recovered from the appropriate fractions as a yellow solid by lyophilization: yield, 0.6 mg (40%); LRMS (ESI), *m*/*z* 1023.2 $(M + H)^+$; theoretical, *m*/*z* 1023.2.

5′-Phosphorylation of Synthetic Oligonucleotide Primers. Reaction mixtures (20 μL total volume) containing 100 pmol of primer, 1 mM ATP, 70 mM Tris at pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1 unit of T4 polynucleotide kinase were incubated at 37 °C for 1 h and then chilled in ice. Then, 40 μL of deionized water, 240 μL of 5 M NH_4-OAc, and 750 μL of cold ethanol were added, and then the combined solution was mixed and incubated at −20 °C for 20 min and centrifuged at 12000*g* for 20 min. The solid product was washed with 80% ethanol, air-dried, and dissolved in 50 μL of RNase-free water. The purity of each primer was analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE) in Tris−boric acid−ethylenediaminetetraacetic acid (EDTA) (TBE) buffer at pH 8.4 containing 89 mM Tris, 89 mM boric acid, and 20 mM EDTA. For the preparation of 5′-^{32}P-labeled primers, 1 μCi of [γ-^{32}P]ATP was added instead of ATP.

Preparation of Misacylated Suppressor tRNA$^{Phe}_{CUA}$s. The ligation reaction was carried out in 50 mM Na *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) at pH 7.5 containing 0.5 mM ATP, 15 mM $MgCl_2$, 20% dimethylsulfoxide, 0.5 μg/μL $tRNA_{CUA}$, 0.01 A_{260} unit/μL of NVOC-protected aminoacyl-pdCpA and 2 units/μL of T4 RNA ligase. After incubation at 37 °C for 60 min, suppressor tRNAs were precipitated by the addition of cold ethanol (0.1 volume of 3 M sodium acetate at pH 4.5 and 3.0 volumes of ethanol), collected by centrifugation, washed with 70% ethanol, and dried. Samples of suppressor tRNAs were dissolved in RNase-free water and deprotected by the use of a 500 W mercury−xenon lamp (3 min at 2 °C).

Construction of pUC-rrnB Plasmid. Plasmid pNot, containing the rrnB gene with one point mutation in 23S rRNA gene (A1067T), was digested with endonucleases *Kpn*I and *Bam*HI. Plasmid pAltex Ex2, having tetracycline resistance, was modified by site-directed mutagenesis to insert a *Kpn*I site. The modified plasmid was selected by restriction analysis and used for the first stage of cloning. The *Kpn*I−*Bam*HI fragment, containing the rrnB operon, was initially incorporated in the multicloning site of modified pAltex-Ex-2. Selection for resistance to tetracycline and sensitivity to ampicillin afforded a plasmid having the rrnB gene. This plasmid and pUC18 were digested with endonucleases *Bam*HI and *Kpn*I, mixed in a 3:1 molar ratio, and treated with T4 DNA ligase (1 h at room temperature). The product of the reaction was transformed into JM-109-competent cells and selected on Luria−Bertani broth (LB) agar, supplemented with ampicillin, X-gal, and isopropyl-β-D-thiogalactopyranose (IPTG). Plasmids from white colonies were purified and analyzed by electrophoresis in a 1% agarose gel after restriction by endonucleases *Kpn*I, *Bam*HI, and *Eco*RI. The sequence of the resulting plasmid was confirmed by partial sequencing in the region of the rrnB operon.

Site-Directed Mutagenesis of the rrnB Operon. Mutagenesis of the rrnB operon was carried out using a QuickChangeTN site-directed mutagenesis kit. The (randomized) primers 5′-ATAAAAGGTACTCCGGGHBVBAHAGGCTGATACCGCCC-3′ and 5′-CCGGGGATAACAGGCVHBVADCGCCCAAGAGTTCATA-3′ were used for positions corresponding to PTC (2447−2450) and helix 89 (2457−2462) of 23S rRNA, respectively (B = C + G + T; H = C + A + T, and V = A + G + C).

Polymerase chain reaction (PCR) was carried out in a 50 μL reaction mixture containing 300 ng of template, 14 pmol of primer, 10 nmol of dNTPs, 2.5 units of Pfu polymerase, and 20 units of Taq DNA ligase in 35 mM Tris-HCl at pH 8.0 containing 12 mM K acetate, 5 mM DTT, 0.05% Triton X-100, and 0.05 mM EDTA. The thermal cycler was programmed as follows: preincubation at 95 °C for 2 min, 18 cycles at 95 °C for 1 min, 42 °C for 1 min, and 65 °C for 24 min. Then, samples were incubated at 75 °C for 7 min and allowed to cool to room temperature. A total of 1 μL of restriction endonuclease *Dpn*I was added, and the reaction mixture was incubated at 37 °C for 1 h. Then, samples were subjected to denaturation at 95 °C for 1 min, followed by 2 cycles at 95 °C for 1 min, 42 °C for 1 min, and 70 °C for 24 min. The samples were then precipitated by the addition of Na acetate at pH 5.2 to a concentration of 0.1 M and 3 volumes of cold ethanol (20 min at −20 °C), centrifuged, washed with 70% ethanol, air-dried, and dissolved in 10 μL of deionized water.

JM-109-competent cells were transformed with 5 μL of mutagenesis mixture, plated on agar that had been prepared

with LB medium supplemented with ampicillin, and incubated at 37 °C overnight. The colonies were transferred to a nitrocellulose filter and grown at 37 °C overnight, and then replicates were prepared on fresh filters. The selection of colonies was further carried out on LB agar, supplemented with ampicillin (100 μg/mL), IPTG (500 μM), and chloramphenicol at concentrations of 5, 10, and 20 μg/mL. One filter was plated on LB agar only with ampicillin and IPTG as a control. Plates were incubated at 37 °C for 48 h. Colonies that grew in the presence of chloramphenicol were transferred to 3 mL of LB medium containing ampicillin and IPTG and grown at 37 °C overnight. Growth rate determination (doubling time) was carried out for all cultures, diluted by fresh medium to 0.03−0.05 OD_{450}. A culture with wild-type plasmid pUCrrnB was tested simultaneously as a control. Plasmids from chloramphenicol-resistant colonies, which demonstrated less than the control doubling time, were purified and sequenced.

S-30 Preparation. S-30 systems from *E. coli* BL-21 (DE-3) lacking and harboring plasmids with (wild-type or mutant) rrnB genes were prepared by the procedure described by Pratt (*34*) with slight modification. *E. coli* BL-21 (DE-3) cells, transformed by the plasmids with wild-type and mutant rrnB operons, were grown at 37 °C in LB medium, supplemented with ampicillin (100 μg/L), until the optical density at 450 nm was 0.5−1.0. They were then diluted 10 times with LB medium supplemented with ampicillin (100 μg/L) and IPTG (500 μM) and again grown at 37 °C, until the optical density at 450 nm was 1.5−2.0. The cells were collected by centrifugation, washed, and pressed at 8400 psi in a French cell press. In a small-scale variation (100−500 mL culture), egg lysozyme (150 μg/g of wet cells) was used instead of pressure for cell rupture. Finally, the S-30 extracts were dialyzed, centrifuged, frozen, and stored at −80 °C.

Preparation of rRNA Fraction from S-30 Systems. A total of 1 mL of the S-30 system was diluted 4 times using 80 mM Hepes-KOH at pH 7.6 containing 24 mM $MgCl_2$, 2 M NH_4Cl, and 20 mM β-mercaptoethanol. Samples were loaded onto 5 mL of sucrose cushion (20% sucrose in 20 mM Hepes-KOH at pH 7.6 containing 6 mM $MgCl_2$, 500 mM NH_4Cl, and 4 mM β-mercaptoethanol). Ribosome particles were centrifuged at 55000*g* for 10 h.

Purification of the rRNA was carried out using a commercial RNAgents total RNA isolation system (Promega, Madison, WI) and analyzed on a 1% agarose gel containing 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer at pH 7.0, 4 mM NaOAc, 0.5 mM EDTA, and 1 M formaldehyde. Samples of RNA (30−50 μg) were diluted with 0.02 M MOPS buffer, supplemented with 50% formamide, 4.5 M formaldehyde, 10% glycerol, 0.025% bromphenol blue, and 0.025% xylene cyanol, and loaded on the gel. Electrophoresis was carried out at 5 V/cm for 3 h in 0.02 MOPS buffer at pH 7.0 containing 80 mM NaOAc and 1 mM EDTA. The gel was washed with deionized water (3 times during 20 min) and stained with ethidium bromide (0.5 μg/mL) for 10 min.

Primer Extension Method. The reaction mixture (5 μL total volume) containing 9 μg of rRNA, 15 ng of [5′-^{32}P]-TTCGTCGGTAGTAAATTTCTTT primer, 100 mM KCl, and 50 mM Tris at pH 8.5 was incubated at 90 °C for 1 min and cooled slowly (∼1 h) to 30 °C. A total of 1 μL of reverse transcriptase buffer, containing 250 mM Tris at pH 8.5, 250 mM KCl, 50 mM DTT, and 50 mM $MgCl_2$, and 1 unit of reverse transcriptase were added to the annealing mixture. Then, 3 μL of dNTP mixture containing 80 μM dATP, dGTP, dCTP, and ddTTP were added. The reaction mixture was incubated at room temperature for 5 min. After incubation, 10 μL of 80% formamide, containing 10 mM EDTA, 0.08% xylene cyanol, and 0.08% bromphenol blue, was added. Primer extension reactions were analyzed by 20% PAGE in 0.1 M Tris−75 mM borate−1.25 EDTA buffer at pH 8.4.

Northern Blot Analysis. After agarose gel electrophoresis, samples of rRNA were transferred onto nylon membranes in 7.5 mM NaOH overnight at room temperature. Nylon membranes with RNA were prehybridized at 37 °C for 1−2 h in 50 mM Na phosphate buffer at pH 7.4 containing 6 mM EDTA, 20% formamide, 0.1% sodium dodecyl sulfate (SDS), 0.4 g/L Ficol Type 400, 0.4 g/L polyvinylpyrrolidone, and 0.4 g/L bovine serum albumin (BSA). 5′-[^{32}P]Oligonucleotide probe (GCCC**ACCG**TGTC), complementary to the mutant regions of clone A4, was added to the prehybridization filter to a specific activity of about 10^8 cpm/μg and incubated at 37 °C for 24 h. The nylon membranes were washed with 7.5 mM Na citrate buffer at pH 7.2 containing 75 mM NaCl and 0.1% SDS and then 3 times for 20 min with 15 mM Na citrate buffer at pH 7.2 containing 150 mM NaCl and 0.1% SDS. The nylon membranes were air-dried and analyzed using a phosphorimager.

Construction of Plasmids with E. coli Dihydrofolate Reductase (DHFR) Genes. pETDHwt, $pETDH_{10}$, and $pETDH_{22}$ were prepared by recloning the corresponding DHFR genes from plasmids pTHD, $pTHD_{22}$, and $pTHD_{10}$, prepared previously (*33*) in pET28b vector. The *Nco*I−*Not*I fragments, containing the DHFR genes, were excised from the plasmids and purified by low-melting agarose gel electrophoresis. Vector pET28b was digested by the same endonucleases, mixed with the fragment in a 1:5 molar ratio, and treated with T4 DNA ligase (1 h at room temperature). After inactivation of the ligase (2 min at 70 °C), the ligation mixture was transformed into *E. coli* JM-109 and grown overnight at 37 °C on LB agar, supplemented with kanamycin. The colonies were recloned on fresh LB agar, supplemented with kanamycin. Plasmids from individual colonies were purified and analyzed by 1% agarose gel electrophoresis.

Plasmids $pETDH_{42}$ and $pETDH_{54}$ were prepared from plasmid $pETDH_{wt}$ by the use of the mutagenesis technique described above. 5′-Phosphorylated oligonucleotides 5′-CCTTAAATAAACCCGTGATT**TAG**GGCCGC-CATACCTGG-3′ and 5′-AATCAATCGGTCGTCCG**TAG**-TACCAGGACGCAAAAATATT-3′ were used for incorporation of a TAG codon in positions corresponding to Met 42 and Leu 54, respectively.

Construction of Plasmids Having Photinus pyralis Firefly Luciferase Genes. A DNA fragment containing the luciferase gene was excised from $pTrcLuc_{wt}$ plasmid (*35*) using *Hin*dIII and *Nco*I restriction endonucleases and purified by low-melting agarose gel electrophoresis. Recloning in pET28b was carried out as described above. The final plasmid was named $pETLuc_{wt}$. Plasmids $pETLuc_{247}$ and $pETLuc_{250}$ were prepared by site-directed mutagenesis. 5′-Phosphorylated synthetic oligonucleotides were used for altering the TTT codons in the positions corresponding to Phe 247 and Phe

250 to TAG (5′-GTTCCATTCCATCACGGT**TAG**GGAATGTTTACTACACTCG-3′ and 5′-CATCACGGTTTGGAATG**TAG**ACTACACTCGGATATTTGATA-3′, respectively).

In Vitro Protein Translation Reaction. Translation reactions were carried out in 20−200 μL reaction mixtures containing 0.1−0.4 μL/μL of S-30 preparations, 40−100 ng/μL of plasmids containing the relevant (wild-type or mutant) genes, 35 mM Tris acetate at pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% poly(ethylene glycol) (PEG)-6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 μM mixture of amino acids, and either 0.5 μCi/μL of ^{35}S-methionine or 1 μCi/μL ^{3}H-amino acid mixture (Leu, Lys, Phe, Pro, and Tyr). In the case of plasmids having genes with the TAG codon, suppressor tRNAs were added to a concentration of 0.3 μg/μL. *In vitro* translation was carried out in the presence of rifampicin (10 μg/mL) to prevent further mRNA synthesis from plasmids containing the mutant rrnB operon (Lac promoter). All plasmids of interest contained DHFR and luciferase genes under the control of a T7 promoter, which was not affected by rifampicin. Reactions were carried out at 37 °C for 1 h (DHFR) or at 30 °C for 1 h (luciferase) and terminated by chilling on ice. Aliquots from *in vitro* translation mixtures were analyzed by SDS−PAGE, and quantification of the band corresponding to the synthesized protein was carried out by phosphorimager analysis. Suppression efficiency was defined as the percent of protein produced via the nonsense suppression reaction relative to the protein production from wild-type mRNA.

Purification of Proteins Synthesized In Vitro. Samples of proteins, prepared by *in vitro* translation, were diluted 5-fold with 50 mM Na phosphate at pH 8.0 containing 0.3 M NaCl and 0.1 mg/mL BSA and applied to 50 μL Ni−NTA agarose columns that had been equilibrated using the same buffer. The columns were then washed successively with three 500 μL portions of the same buffer and one 500 μL portion of the same buffer supplemented with 20 mM imidazole. Elution of the bound proteins was carried out using 150 μL of 50 mM Na phosphate at pH 8.0 containing 0.1 mg/mL BSA and 150 mM imidazole. The amount of ^{35}S- (or ^{3}H-) labeled protein was determined by a liquid scintillation counter and confirmed by SDS−PAGE analysis and quantification using a phosphoimager.

Quantitation of the Enzymatic Activity. The enzymatic activity of DHFR was determined by the method of Baccanari et al. (*36*). Reaction mixtures (1 mL total volume) containing 50 mM 4-morpholinemethanesulfonate (MES), 25 mM Tris at pH 7.0, 25 mM ethanolamine, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 100 mM dihydrofolic acid, and 100 mM NaDPH were incubated at 37 °C for 1−2 min in a thermostatted cuvette (1 cm light path). Aliquots (5−10 μL) of samples were added, and the optical density at 340 nm was measured over a period of 10 min. A total of 1 unit of DHFR was defined as the amount of enzyme required to reduce 1 μM dihydrofolic acid/min at 37 °C and pH 7.0 (molar extinction coefficient = 11×10^{-3} M^{-1} cm^{-1}).

The enzymatic activity of luciferase was determined using a commercial luciferase assay system (Promega). Samples (10 μL) were added to 100 μL of luciferase assay reagent, and light emission was measured immediately. Enzyme activity was expressed as the level of light emission (Em) at the maximum wavelength (λ_{max}).

RESULTS

In the present work, modified ribosomes were obtained by the overexpression of modified 23S rRNA from multicopy plasmids. The modified ribosomes had mutations in two regions (2447−2450 and 2457−2462) of *E. coli* 23S rRNA; these correspond to the PTC and helix 89, respectively. The latter region has been found to play an important role in translation by binding the 3′-(CCA) end of aminoacyl-tRNA (*37*).

Construction of Plasmids with the rrnB Operon, Containing Mutations in Two Regions of Domain V of 23S rRNA. Plasmid pUCrrnB, containing the wild-type rrnB operon under the control of an IPTG-induced Lac promoter, was constructed using high copy number vector pUC18 and the rrnB gene (Figure 1). The latter had a point mutation in the 23S rRNA (A1067T), which is responsible for the resistance of the ribosome to the antibiotic thiostrepton (*38*). The presence of this mutation allowed differentiation between chromosomal- (wild-type) and plasmid-borne ribosomes during *in vitro* translation. To prepare the modified ribosomes, nucleotides in two regions of the 23S rRNA gene of pUCrrnB plasmid (PTC and helix 89) were changed by the use of site-directed mutagenesis (Figure 1).

Cells with mutant ribosomes are known to have a diminished growth rate (*29*, *30*). Therefore, to increase the yield of the mutants, special random primers lacking wild-type nucleotides in the modified positions were used in the mutagenesis procedure (Figure 1). Also, to permit the selection of cells having mutant ribosomes, two selection steps were used. The regions of 23S rRNA chosen for mutagenesis have been demonstrated to be responsible for chloramphenicol binding (*29*, *38*). Therefore, this antibiotic was used for the initial selection of mutants. Cells putatively containing mutant ribosomes were grown in the presence of different chloramphenicol concentrations, and the doubling time of the surviving clones was compared with cells containing plasmid pUCrrnB. Those clones having altered doubling times were presumed to have alterations in the nucleotide regions of interest. After the plasmids were sequenced from chloramphenicol-resistant clones with a decreased growth rate, eight mutant rrnB genes were obtained (two in the region 2447−2451 and six in the region 2457−2462) (Table 1).

Cell-Free Protein-Synthesizing System with Modified Ribosomes. The influence of mutations in 23S rRNA on protein synthesis and incorporation of D-amino acids into proteins was studied by the use of cell-free protein-synthesizing systems (S-30s) prepared from *E. coli* cell cultures containing plasmids with wild-type or mutant rrnB genes. The BL-21 (DE-3) strain was chosen for S-30 preparation. This strain contains mutations in the genes responsible for some endopeptidase, proteinase, and exonuclease activities, resulting in the stability of templates and expressed proteins. The presence of T7 RNA polymerase permitted mRNA synthesis to be limited to those genes under the control of the T7 promoter by the use of rifampicin. The level of plasmid-borne ribosomes in the mutant S-30 preparations was estimated as the level of protein synthesis *in vitro* in the

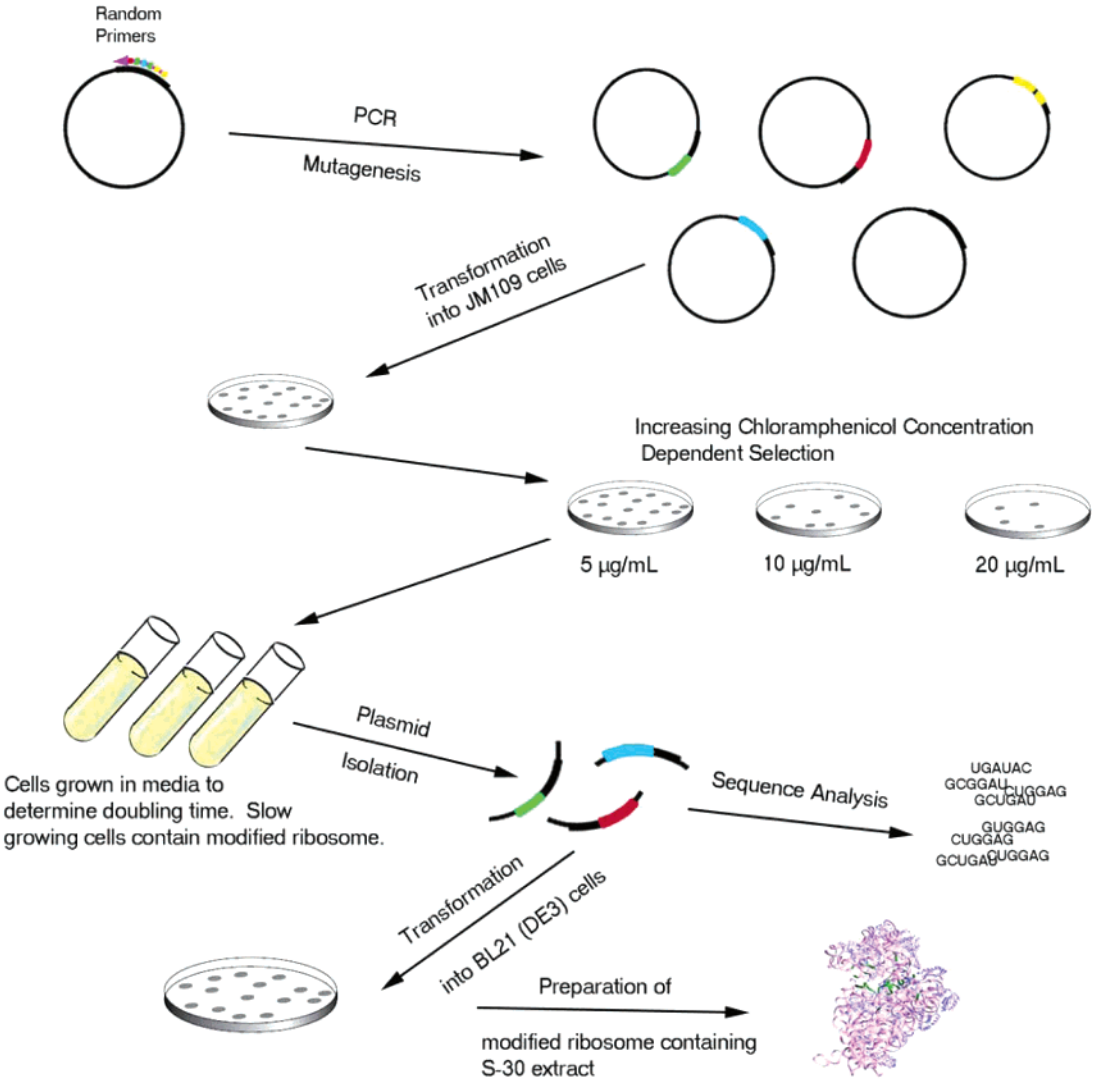


FIGURE 1: Strategy for the construction of modified ribosomes capable of utilizing D-amino acids in protein synthesis.

Table 1: Sequence of Plasmids from Colonies after Mutagenesis in Two Regions of 23S rRNA[a]

clones	sequence in region 2447−2450	sequence in region 2457−2462
wild type	GATA	TGATAC
A3	**TTGT**	TGATAC
A4	**TGGC**	TGATAC
B14	GATA	**GCGGAT**
B23	GATA	**CTGGAG**
B24	GATA	**CTGGAG**
B25	GATA	**GCTGAT**
B39	GATA	**GTGGAG**
B40	GATA	**GCCGAT**

[a] Mutant sequences in boldface type.

presence of thiostrepton (Table 2). Some clones were verified by primer extension. The percentage of mutant ribosomes varied from 36 to 63% (and correlated with control S-30 containing wild-type plasmid-borne ribosomes).

The presence of the expected mutations in the 23S rRNA fraction of modified S-30 preparations was confirmed by the use of Northern blotting (Figure 2). Samples of rRNA from the ribosome fraction of three different S-30 systems without and with the mutation 2447UGGC2450 were isolated and hybridized with a DNA probe complementary to the mutant region. As seen from Figure 2, the DNA probe bound only to the 23S rRNA from the mutant S-30.

Additionally, the chloramphenicol resistance of S-30 preparations with modified ribosomes was checked. Translation of wild-type DHFR in the presence of chloramphenicol was carried out using three different S-30 preparations (one lacking and two containing modified ribosomes) (Figure 3). As seen in Figure 3, both of the tested modified S-30 preparations supported *in vitro* protein synthesis in the presence of 10 μg/mL chloramphenicol.

Study of the Activity and Fidelity of S-30 Preparations Containing Modified Ribosomes. The efficiency of translation in S-30 systems containing modified ribosomes was determined by carrying out *E. coli* DHFR synthesis from the wild-type gene. The yield of proteins from 1 mL of translation mixture in the case of S-30 preparations containing modified and unmodified plasmid-borne ribosomes varied from 12 to 40 μg and was about 3−6 times less than in the case of the S-30 system lacking plasmid-borne ribosomes (Table 2). Therefore, all translation experiments were carried out using normalized amounts of S-30 preparations.

The translation fidelity of the modified ribosomes was estimated using two enzymes, *E. coli* DHFR and *P. pyralis* firefly luciferase. Thiostrepton (2.3 μg/mL) was added to

Table 2: Characteristics of Cell-Free Protein-Synthesizing Systems Having Modified Ribosomes[a]

S-30 preparation	type of plasmid-borne ribosome	sequence in regions 2447−2450	sequence in regions 2457−2462	level of plasmid-borne ribosome[b] (%)	yield of DHFR (μg/mL)
1	none	GAUA	UGAUAC	no	102
2	wild type	GAUA	UGAUAC	45 (64)	36
3	mutant	**UUGU**	UGAUAC	36 (29)	25
4	mutant	**UGGC**	UGAUAC	43 (34)	26
5	mutant	GAUA	**GCGGAU**	52	13
6	mutant	GAUA	**CUGGAG**	55	23
7	mutant	GAUA	**GCUGAU**	63 (56)	12
8	mutant	GAUA	**GUGGAG**	40	15

[a] Mutant sequences in boldface type. [b] Determined as a percentage of the wild-type DHFR synthesis in the presence of thiostrepton, relative to DHFR synthesis in the absence of this antibiotic; data in parentheses obtained by the primer extension method.

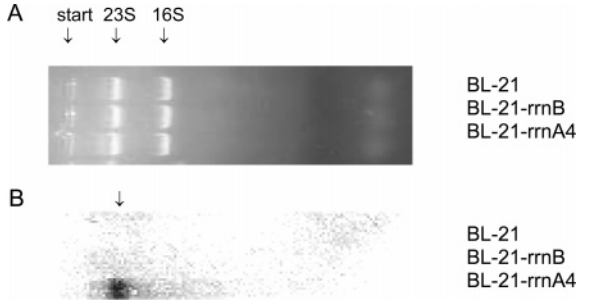


FIGURE 2: Northern blot analysis of S-30 systems: BL-21, chromosome-borne ribosomal RNA; BL-21-rrnB, wild-type chromosome- and plasmid-borne ribosomal RNA; and BL-21-rrnA4, wild-type chromosome-borne and mutant (2447UGGC2450) plasmid-borne ribosomal RNA. (A) Agarose gel before transfer to the nylon membrane. (B) Nylon membrane with samples of rRNA after incubation with a specific DNA probe.

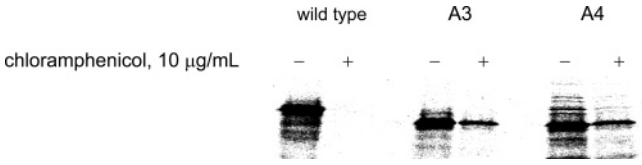


FIGURE 3: Study of chloramphenicol resistance of S-30 systems with additional mutant ribosome: wt, S-30 preparation containing wild-type ribosomes; A3, S-30 preparation containing ribosomes with mutation (2447UUGU2450) in 23S rRNA; and A4, S-30 preparation containing ribosomes with mutation (2447UGGC2450) in 23S rRNA. Separation of synthesized proteins was carried out by PAGE; the proteins were analyzed using a phosphorimager.

the translation mixture to block the action of the chromosome-borne ribosome (95−98% inhibition). The relative activity of the elaborated enzymes was compared with control samples, synthesized by an S-30 preparation containing wild-type ribosomes. It was shown that the mutant ribosomes could synthesize active DHFR and luciferase (77−85 and 58−75% of wild-type activity, respectively) (Table 3). Thus the modified ribosomes produced wild-type proteins that apparently contained some microheterogeneity as a consequence of misincorporation of proteinogenic amino acids.

Synthesis of D*-Methionyl and* D*-Phenylalanyl-tRNAs.* Commercially available D-methionine and D-phenylalanine were first treated with L-amino acid oxidase (*39*) to destroy any residual L isomers. D-Methionine was treated with NVOC chloride in dioxane in the presence of sodium carbonate to afford the *N*-NVOC-D-methionine (**1**, Scheme 1) in moderate yield. The conversion of **1** to the corresponding cyanomethyl ester was achieved by treatment with chloroacetonitrile in dry acetonitrile in the presence of triethylamine. Cyanomethyl ester **2** was obtained in 62% yield. The treatment of active ester **2** with a solution of tris-(tetrabutylammonium) salt of pdCpA in dry DMF gave the corresponding aminoacylated pdCpA (**3**) in 36% yield.

Table 3: Translation Fidelity of S-30 Preparations Containing Mutant Ribosomes[a]

type of plasmid-borne ribosome	sequence in 23S rRNA regions 2447−2450	sequence in 23S rRNA regions 2457−2462	relative enzyme activity (%) luciferase	relative enzyme activity (%) DHFR
wild type	GAUA	UGAUAC	100	100
mutant	**UGGC**	UGAUAC	58 ± 10	82 ± 6
mutant	GAUA	**GCGGAU**	62 ± 7	77 ± 5
mutant	GAUA	**GCUGAU**	75 ± 6	85 ± 2

[a] Mutant sequences in boldface type.

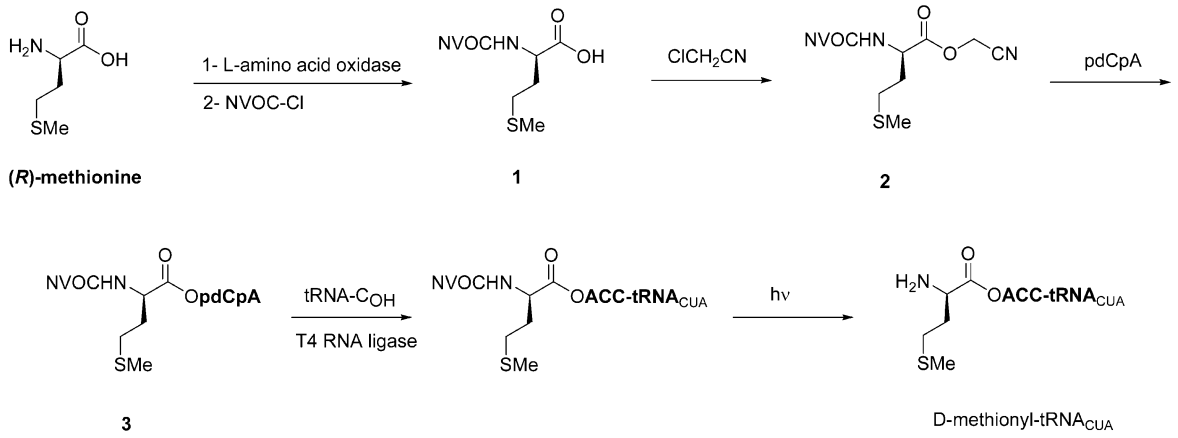
In a similar manner, D-phenylalanine was dissolved in aqueous dioxane and treated with NVOC chloride (Scheme 2). The N-protected D-phenylalanine (**4**) was obtained in 57% yield and activated as the cyanomethyl ester (**5**) by treatment with triethylamine and an excess of chloroacetonitrile. Activated ester **5** was coupled to pdCpA in DMF, and the product was isolated by HPLC, affording **6** in 40% yield.

Aminoacylated dinucleotides **3** and **6** were used to prepare D-methionyl- and D-phenylalanyl-tRNA$_{CUA}$s, respectively, as shown in Schemes 1 and 2.

Study of the Influence of Ribosome Modifications on UAG Codon Suppression in the Presence of D*-Aminoacyl-tRNAs.* Plasmid pETDH$_{22}$ (containing a TAG codon in the position corresponding to Trp 22 of the DHFR gene) was used for *in vitro* translation. Translations using control S-30 preparations (without and with wild-type plasmid-borne ribosomes) were carried out simultaneously. The level of protein synthesis in the presence of the L isomers of methionine and phenylalanine was the same in S-30 preparations with and without modified ribosomes (30−50%). However, practically no protein synthesis was observed in the case of the S-30 system lacking plasmid-borne ribosomes in the presence of D-phenylalanyl-tRNA and D-methionyl-tRNA (Tables 4 and 5). As seen in Table 4, most of the modified S-30 preparations demonstrated an enhanced level of suppression of UAG codons, using both D-methionyl and D-phenylalanyl-tRNA$_{CUA}$s (about 17−23 and 9−12%, respectively).

Also studied was the time course of synthesis of full-length proteins elaborated from a mRNA containing a UAG codon

Scheme 1



Scheme 2

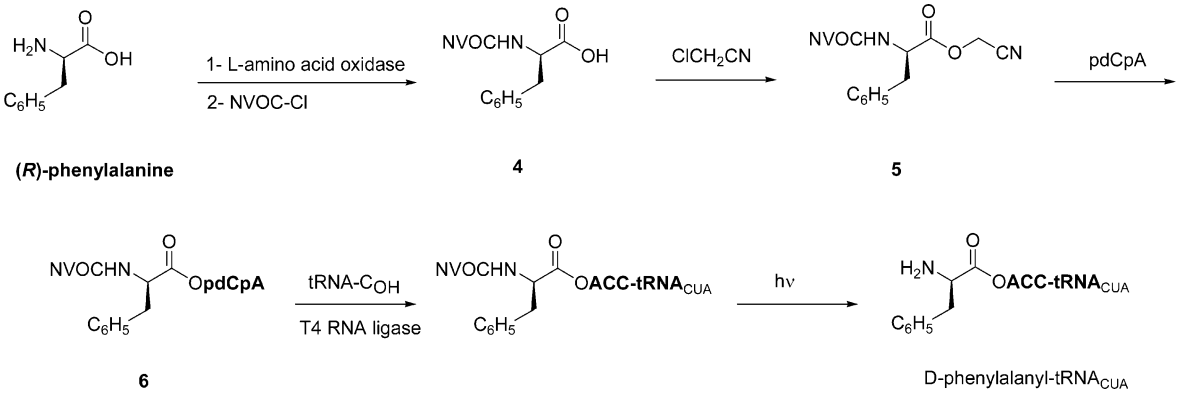


Table 4: Comparison of the Abilities of S-30 Preparations Having Different Kinds of Ribosomes To Suppress UAG Codons in the Presence of D-Methionyl- and D-Phenylalanyl-tRNAs

S-30 preparation	plasmid-borne ribosome	suppression efficiency[a] (%)		
		D-Met-tRNA	D-Phe-tRNA	unacylated tRNA
1	no	5.3 ± 2.1	2.8 ± 0.6	1.8 ± 0.5
2	wild type	10.1 ± 1.5	4.2 ± 1.5	1.4 ± 0.8
3	mutant	20.9 ± 2.7	8.9 ± 1.5	4.3 ± 0.6
4	mutant	22.9 ± 3.0	11.1 ± 2.3	4.0 ± 1.8
5	mutant	17.5 ± 1.8	8.9 ± 0.5	4.5 ± 0.5
6	mutant	16.9 ± 1.6	11.5 ± 0.4	6.4 ± 1.0
7	mutant	19.7 ± 2.4	8.6 ± 2.2	4.8 ± 1.0
8	mutant	11.1 ± 2.3	4.4 ± 0.5	3.0 ± 0.5

[a] Determined as a percentage of full-length DHFR, formed by the readthrough of a UAG codon at position 22, relative to the amount of full-length DHFR produced using wild-type mRNA. For wild-type mRNA, all of the S-30 preparations exhibited comparable levels of DHFR synthesis. Each number represents the average of three independent experiments ± SE.

Table 5: Suppression of a UAG Codon at Position 22 of DHFR mRNA with D- and L-Aminoacyl-tRNA$_{CUA}$s[a]

amino acid	suppression (%)	
	wild-type ribosomes	wild-type + mutant[b] ribosomes
L-phenylalanine	58	54
D-phenylalanine	3	12
L-methionine	52	47
D-methionine	5	23
	0.9	2

[a] Relative to the amount of protein produced using wild-type mRNA and wild-type ribosomes. [b] S-30 preparation 4 (clone A4).

in the presence of D- and L-methionyl-tRNAs. The results are shown for the suppression of a UAG codon at position 22 of DHFR mRNA (Figure 4). As shown, the time course of suppression for the two aminoacyl-tRNAs was quite similar, although suppression by L-methionyl-tRNA$_{CUA}$ was more efficient overall.

Because it has been demonstrated that modified ribosomes, especially those containing mutations in PTC, can read through UAG codons nonspecifically (*29*, *30*), the level of DHFR synthesis from the modified gene in the presence of unacylated tRNA$_{CUA}$ was carefully checked (Table 4). As shown, all analyzed S-30 preparations having mutant ribosomes have demonstrated ~1.5−3-fold greater nonspecific readthrough in comparison with the wild type, but this level was still substantially less than the level of protein synthesis in the presence of D-phenylalanyl and D-methionyl-tRNA$_{CUA}$s.

Study of Mutant Proteins Prepared by In Vitro Translation in the Presence of D-Amino-Acid-Containing Suppressor tRNAs. Two enzymes (*E. coli* DHFR and *P. pyralis* firefly luciferase) were used for these purposes. Four different plasmids with mutant DHFR genes (TAG codon in positions corresponding to Val 10, Trp 22, Met 42, and Leu 54) and two with mutant luciferase genes (TAG codon in positions corresponding to Phe 247 and Phe 250) were prepared. Translation of each of the modified enzymes was carried out in the presence of L- and D-aminoacyl-tRNAs. The corresponding enzymes were also translated from wild-type genes. It was found that the suppression efficiencies obtained with D-methionyl-tRNAs and D-phenylalanyl-tRNAs were

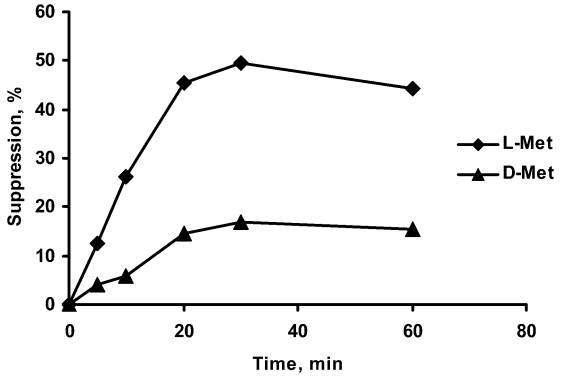


FIGURE 4: Time course of suppression of a UAG codon at position 22 of DHFR in the presence of L- and D-methionyl-tRNA$_{CUA}$s. S-30 preparation of A4 was employed for this experiment.

Table 6: Specific Activities of Proteins Containing D- versus L-Amino Acids at Specific Positions

	specific activity (%)[a]			
	phenylalanine		methionine	
position (wild-type amino acid)	D	L	D	L
DHFR				
10 (valine)			83.7 ± 9.5	92.5 ± 5.8
22 (tryptophan)	13.1 ± 6.9	90.2 ± 4.6		
54 (leucine)			11.3 ± 2.7	56.2 ± 4.4
luciferase				
247 (phenylalanine)	10.2 ± 5.3	89.6 ± 7.4		
250 (phenylalanine)	63.6 ± 9.4	93.2 ± 6.1		

[a] Relative to the same protein prepared using wild-type mRNA in the wild-type cell-free protein-synthesizing system, defined as 100%.

typically 35−45% of those obtained using the respective L-aminoacyl-tRNA$_{CUA}$s. After purification, enzyme activity was tested using equal amounts of samples and compared (Table 6). As seen in Table 6, three modified enzymes containing D-amino acids differed significantly from those containing the same L-amino acid. These included two modified DHFRs (positions 22 and 54), translated in the presence of D-phenylalanyl and D-methionyl-tRNAs, respectively, and one luciferase mutant (position 247), translated in the presence D-phenylalanyl-tRNA. The other two modified enzymes containing D-methionine at position 10 of DHFR and D-phenylalanine at position 250 of firefly luciferase had activities not dramatically different from the enzymes with the respective L-amino acids in those positions.

Peptide Mapping of DHFR Mutants Synthesized in the Presence of Suppressor L- and D-tRNA$_{CUA}$s. Modified DHFRs synthesized from DHFR mRNA containing a UAG codon in the position corresponding to Met 42 in the presence of L-methionyl- and D-methionyl-tRNA$_{CUA}$s were used for the peptide-mapping experiment. Two DHFR samples (wild type and mutant, translated from the same modified DHFR gene in the presence of 2-naphthylalanyl-tRNA$_{CUA}$) were used as positive and negative controls, respectively. All samples were treated with BrCN under acidic conditions and analyzed by high-resolution PAGE (*40*). All samples were ^{3}H-labeled during translation to permit visualization of peptide maps by autoradiography (Figure 4). It was found that both modified DHFRs, prepared in the presence of D- and L-methionyl-tRNAs, gave the same peptide map as the wild-type enzyme. In the case of the control sample having 2-naphthylalanine in position 42, no fragment with a molecular weight of ~6 kDa was observed.

DISCUSSION

The use of purposefully misacylated tRNAs in ribosomally mediated protein-synthesizing systems has enabled the elaboration of proteins containing unnatural amino acids (*16*, *41*). While most of these substitutions have involved simple alteration of the C$^\alpha$ side chain, there are also examples of more fundamental alterations of the amino acid structure (*42*, *43*). The latter support the potential for the preparation of novel biomaterials and the detailed analysis of protein functions but are limited by the specificity of wild-type ribosomes for C$^\alpha$ amino acids having the L configuration. D-Amino acid incorporation is one example of the limitations of the wild-type system. In the present study, a novel strategy involving the modification of the bacterial ribosome was employed to facilitate enhanced incorporation of D-amino acids into proteins at specific sites *in vitro*.

The discrimination between L- and D-amino acids at the level of tRNA activation was avoided by the addition of preformed D-aminoacyl-tRNA to the translation mixtures. It has been demonstrated previously that synthetic yeast tRNA$^{Phe}_{CUA}$-C$_{OH}$, used for the chemical aminoacylation procedure, is not recognized by *E. coli* phenylalanyl-tRNA synthetase and, therefore, cannot be reactivated in an *E. coli* system (*44*).

A set of modified ribosomes having mutations in PTC (2447−2450) and helix 89 (2457−2462) of *E. coli* 23S rRNA was prepared. Both mutants in the region 2447−2450 contained the mutations G2447U and U2449G. According to one of the PTC models (*24*), the O-6 atom of G2447 is critical for a charge relay network with A2451 and A2450. It is possible that the O-4 atom of mutant U2447 can mimic G2447 in this kind of interaction. All mutants in region 2457−2462 had the substitution U2460G. According to the literature, this substitution increases misreading but does not lead to a lethal phenotype (*37*). Three of the six mutants in helix 89 had the same sequence (CUGGAG) instead of UGAUAC (wild-type), and another differed by only one nucleotide (G2457 instead of C2457).

The presence of the expected 23S rRNA mutants in the ribosomes of the prepared S-30s confirmed that the selected sequences are competent for translation and can provide sufficient fidelity, which increased the chance for survival. All S-30 preparations were active in the presence of thiostrepton, which blocked the chromosome-borne ribosome, and were able to synthesize catalytically competent DHFR and luciferase, although the fidelity of protein synthesis was less than that for wild-type S-30 (Table 3). These results are in good agreement with the earlier findings that mutations in the PTC region facilitate readthrough (*29*, *30*). Indeed, our experiments have shown that modified S-30 preparations can read through UAG codons in the absence of suppressor tRNAs to a greater extent than wild-type S-30 preparations (Table 4). However, the ratio of specific (i.e., in the presence of activated suppressor tRNAs) versus nonspecific readthrough

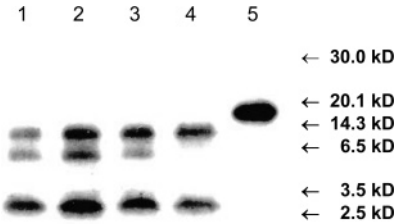


FIGURE 5: BrCN peptide mapping of *E. coli* DHFR samples synthesized *in vitro* from wild-type mRNA (lane 2) and modified mRNA (UAG codon in position 42, corresponding to Met 42 in the wild type) in the presence of L-methionyl-$tRNA_{CUA}$ (lane 1), D-methionyl-$tRNA_{CUA}$ (lane 3), or L-naphthylalalanyl-$tRNA_{CUA}$ (lane 4). Lane 5, control DHFR sample without BrCN treatment. Separation was carried out by PAGE; the proteins were analyzed by autoradiography.

was high enough to permit the use of the modified ribosomes for the study of D-amino acid incorporation into the protein by suppression of UAG codons in the mRNAs. It has been demonstrated that S-30 systems lacking plasmid-borne ribosomes exhibit only slight protein synthesis in the presence of D-methionyl-$tRNA_{CUA}$ and especially D-phenylalanyl-$tRNA_{CUA}$, but using the S-30 preparations containing mutant ribosomes resulted in higher levels of UAG codon suppression in the presence of both D-methionine and D-phenylalanine (Table 4).

A comparison of BrCN peptide mapping of DHFR samples, synthesized in the presence of L- and D-methionyl $tRNA_{CUA}$s from DHFR mRNA containing a UAG codon in the position corresponding to Met 42, has confirmed that the level of nonspecific readthrough is low relative to specific readthrough of the UAG codon (Figure 5). The absence of methionine in position 42 leads to the disappearance of the peptide with a molecular weight of about 6 kDa. As shown in Figure 5, this peptide is absent for the modified DHFR containing L-naphthylalanine in this position. The presence of this peptide in the case of modified DHFRs synthesized with L- and D-methionyl $tRNA_{CUA}$s in the incubation mixtures confirms that both isomers of methionine are incorporated into position 42 during *in vitro* translation.

To exclude the possibility that D → L conversion during *in vitro* translation formed the basis for the observed results, the specific activities of *E. coli* DHFRs and *P. pyralis* firefly luciferases, synthesized from the UAG-containing mRNAs in the presence L- and D-methionyl and L- and D-phenylalanyl-$tRNA_{CUA}$s, were compared (Table 6). Two kinds of positions in these proteins were chosen. The first are known to be important for enzyme function [Trp 22 and Leu 54 for DHFR (*45*, *46*) and Phe 247 for luciferase (*47*)]. The second positions are believed to be less important for supporting enzyme function (Val 10 for DHFR and Phe 250 for luciferase). We found that the presence of D isomers in essential positions of these proteins (D-methionine in position 54 of DHFR and D-phenylalanine in position 22 of DHFR and position 247 of luciferase) resulted in a significant decrease in enzyme activity. In contrast, proteins containing either D- or L-amino acids in less important positions (position 10 for DHFR and position 250 for luciferase) showed much smaller differences in activity.

Thus, we have demonstrated that modified ribosomes can have different translation properties compared to wild-type ribosomes *in vitro.* It has been shown that alterations in some regions of 23S rRNA, responsible for the binding of translation factors and ribosomal proteins, alter the pretranslation state of ribosomes and, as a result, alter the translation properties (*28*, *48*). In the present case, such alterations may facilitate the binding and processing of misacylated D-aminoacyl-$tRNA_{CUA}$s in the A site of the ribosome and enhance the probability of using D-amino acids for *in vitro* protein synthesis.

ACKNOWLEDGMENT

We thank Dr. K. H. Nierhaus for plasmid pNot and Dr. S. V. Mamaev for the plasmid containing the $tRNA^{Phe}_{CUA}$-C_{OH} gene.

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BI060986A