Site-Specific Incorporation of (Aminooxy)acetic Acid into Proteins†

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ABSTRACT: By employing a general biosynthetic method for the elaboration of proteins containing unnatural amino acid analogues, we incorporated (aminooxy)acetic acid into positions 10 and 27 of *Escherichia coli* dihydrofolate reductase. Introduction of the modified amino acid into DHFR was accomplished in an in vitro protein biosynthesizing system by readthrough of a nonsense (UAG) codon with a suppressor tRNA that had been activated with (aminooxy)acetic acid. Incorporation of the amino acid proceeded with reasonable efficiency at codon position 10 but less well at position 27. (Aminooxy)acetic acid was also incorporated into position 72 of DNA polymerase β. Peptides containing (aminooxy)acetic acid have been shown to adopt a preferred conformation involving an eight-membered ring that resembles a γ-turn. Accordingly, the present study may faciliate the elaboration of proteins containing conformationally biased peptidomimetic motifs at predetermined sites. The present results further extend the examples of ribosomally mediated formation of peptide bond analogues of altered connectivity and provide a conformationally biased linkage at a predetermined site. It has also been shown that the elaborated protein can be cleaved chemically at the site containing the modified amino acid.

Site-directed mutagenesis is an important technique used to help to understand the forces that control protein structure, folding, and catalysis (*1*−*3*). This technique makes it possible to replace a specific amino acid in a protein with any of the other 19 amino acid building blocks. In contrast to studies of small bioactive molecules, for which analogues with virtually any structural change can be prepared by chemical synthesis, changes in protein structure achieved using site-directed mutagenesis are limited to the 20 amino acids that normally occur in proteins. Ideally, it would be beneficial to be able to modify, e.g., the steric or electronic properties of an amino acid in a protein to address a specific structure−function question. Other modifications of interest might include modification of the acidity, length, hydrogen-bonding properties, or nucleophilicity of the amino acid side chain. In recent years, it has become possible to elaborate such modified proteins by utilizing misacylated suppressor tRNAs (*4*−*9*) to effect the readthrough of nonsense (*10*−*28*) or other unique (*29*, *30*) codons.

Peptidomimetics also offer a unique opportunity to dissect and investigate structure−function relationships in peptides and complex proteins (*31*, *32*). The imposition of conformational constraints can significantly aid in determinations of this type (*33*), and the elaboration of such motifs for introduction into (poly)peptides has been addressed by chemical synthesis (*34*−*36*). In principle, it should be possible to incorporate some peptidomimetic motifs into proteins by ribosomally mediated protein biosynthesis, provided that the ribosome is capable of creating polypeptide analogues of altered connectivity. In fact, some examples of the ability of the ribosome to elaborate peptide analogues have been documented (*37*−*39*).

Presently, the ability of (aminooxy)acetic acid (Scheme 1) to function as an amino acid surrogate in protein synthesis has been studied. The inclusion of (aminooxy)acetyl-tRNA$_{CUA}$ in a protein biosynthesizing system programmed with dihydrofolate reductase mRNAs containing the nonsense codon UAG at a defined position resulted in the incorporation of this amino acid analogue into DHFR.[1] In addition to increasing the repertoire of substrates shown to be capable of forming peptide bond analogues in a ribosomal system, this observation is of interest in the context of proteins bearing peptidomimetic motifs having a defined conformational bias. Peptides containing (aminooxy)acetic acid have been shown to form an N−O turn (*40*−*42*), and the incorporation of this species into appropriate sites in proteins may permit analogous stabilization of structure by intramolecular hydrogen bonding (*43*, *44*). In addition to permitting an evaluation of the effects of replacing structural domains whose flexibility is putatively essential to protein function with a motif that lacks such flexibility, the N−O bond itself is amenable to site-specific cleavage with a variety of (bio)-chemical reagents (*45*−*49*), thereby providing an additional tool for analysis of protein function.

EXPERIMENTAL PROCEDURES

General Methods and Materials. [^{35}S]Methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from Amersham Life

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[1] Abbreviations: DHFR, dihydrofolate reductase; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DEPC, diethyl pyrocarbonate; FABMS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; LB, Luria broth; THF, tetrahydrofuran; NVOC, 6-nitroveratryloxycarbonyl; PAGE, polyacrylamide gel electrophoresis; WT, wild type; BSA, bovine serum albumin; pdCpA, 5′-phosphorylated 2′-deoxycytidylyl(3′→5′)adenosine.

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Science (Arlington Heights, IL). Nuclease-treated rabbit reticulocyte lysate was obtained from Promega Corp. (Madison, WI). Restriction endonucleases and purified acylated bovine serum albumin (BSA) were from New England Biolabs (Beverly, MA), while AmpliScribe transcription kits and T7 RNA polymerase were purchased from Epicentre Technologies (Madison, WI). Acrylamide, *N*,*N*-methylenebis(acrylamide), urea, Tris base, DEAE-Sepharose CL-6B, (aminooxy)acetic acid hemihydrochloride, diethyl pyrocarbonate, and other chemicals were from Sigma Chemicals (St. Louis, MO). Plasmid DNAs were isolated using a Jet-Star midi plasmid preparation kit from PGC Scientific (Gaithersburg, MD). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was carried out using the standard Laemmli procedure (*50*). Gels were visualized and quantified utilizing a Molecular Dynamics 400E phosphorimager equipped with ImageQuant version 5.0 software. All procedures involving water employed distilled, deionized water from a Milli-Q system. UV spectral measurements were made using a Perkin-Elmer Lambda 20 UV/vis spectrometer. Radioactivity measurements were performed with a Beckman LS-100C liquid scintillation counter. Radioactivity measurements were also made using a calibrated phosphoimager screen, such that the pixel density of the image could be directly related to the amount of radioactivity in the sample.

*N-NVOC-(aminooxy)acetic Acid (**1**).* To a solution of 1 mL of water containing 83 mg (0.78 mmol) of Na_2CO_3 was added 44 mg (0.40 mmol) of (aminooxy)acetic acid hemihydrochloride. After being cooled in an ice bath, a solution containing 144 mg (0.52 mmol) of NVOC chloride in 2 mL of dioxane was added, and the reaction mixture was maintained at 0 °C. The reaction was complete after 4 h, as judged by analysis on silica gel TLC. The dioxane was removed under diminished pressure, and the residue was acidified with 1 mL of 1 N $NaHSO_4$. The reaction mixture was extracted with ethyl acetate, and the organic phase was concentrated and applied to a silica gel column (40 × 2 cm); elution was with 20:1 ethyl acetate−acetic acid (silica gel TLC R_f 0.2 with 20:1 ethyl acetate−acetic acid). The solvent was concentrated under diminished pressure, affording *N*-NVOC-(aminooxy)acetic acid (**1**) as a yellow foam: yield 69 mg (52%); ^{1}H NMR ($CDCl_3$) δ 3.96 (m, 6H), 4.49 (m, 2H), 5.60 (m, 2H), 6.95 (s, 1H), and 7.70 (s, 1H); mass spectrum (FAB) *m*/*z* 331.0786 [$(M + H)^+$] ($C_{12}H_{15}NO_9$ requires *m*/*z* 331.0779).

*N-NVOC-(aminooxy)acetic Acid Cyanomethyl Ester (**2**).* To a solution containing 68 mg (0.26 mmol) of NVOC-(aminooxy)acetic acid (**1**) in 20 mL of dry acetonitrile at room temperature were added 296 μL (199 mg, 1.5 mmol) of *N*,*N*-diisopropylethylamine and 65 μL (78 mg, 1.5 mmol) of chloroacetonitrile. The reaction mixture was stirred under nitrogen for 6 h. The solvent was concentrated under diminished pressure, and the residue was redissolved in 10 mL of ethyl acetate. The solution was extracted with saturated aqueous $NaHCO_3$. The organic layer was dried with Na_2SO_4 and applied to a silica gel column (30 × 3 cm). Elution with 50:50:1 EtOAc−hexanes−HOAc afforded fractions containing the desired product. The fractions were coevaporated with portions of toluene to effect removal of acetic acid, affording *N*-NVOC-(aminooxy)acetic acid cyanomethyl ester (**2**) as a yellow solid: yield 17 mg (24%); ^{1}H NMR ($CDCl_3$) δ 3.96 (s, 3H), 3.98 (s, 3H), 4.58 (s, 2H), 4.83 (s, 2H), 5.60 (s, 2H), 7.00 (s, 1H), and 7.72 (s, 1H); mass spectrum (FAB) *m*/*z* 369.0822 (M^+) ($C_{14}H_{15}N_3O_9$ requires 369.0836).

*N-NVOC-(aminooxy)acetyl-pdCpA (**3**).* To a 1 mL screw-cap vial equipped with a stir bar and containing 13.5 mg (34 μmol) of NVOC-(aminooxy)acetic acid cyanomethyl ester (**2**) was added 5.0 mg (3.7 μmol) of (tetrabutylammonium)-pdCpA. Fifty microliters of DMF was added through a rubber septum. The course of the reaction was monitored by HPLC using a C_{18} reversed-phase column (250 × 10 mm); 1 μL aliquots of the reaction mixture were diluted with 50 μL of 50 mM NH_4OAc and used for analysis. The column was washed with 1% → 63% acetonitrile in 50 mM NH_4-OAc, pH 4.5, over a period of 40 min at a flow rate of 3.5 mL/min (detection at 260 nm). After the reaction was judged to be complete, the reaction mixture was quenched by the addition of 200 μL of 1:2 acetonitrile−NH_4OAc (50 mM), pH 4.5, and purified by HPLC using the same conditions described above. The products had retention times of 17.3 and 17.9 min, for the two positional (2′,3′) isomers. After lyophilization of the appropriate fractions, *N*-NVOC-(aminooxy)acetyl-pdCpA (**3**) was obtained as a colorless solid: yield 3 mg (60%); λ_{max} 260 nm (pH 4.5); mass spectrum (FAB) *m*/*z* 949.1783 [$(M + H)^+$] ($C_{31}H_{39}N_{10}O_{21}P_2$ requires *m*/*z* 949.1768).

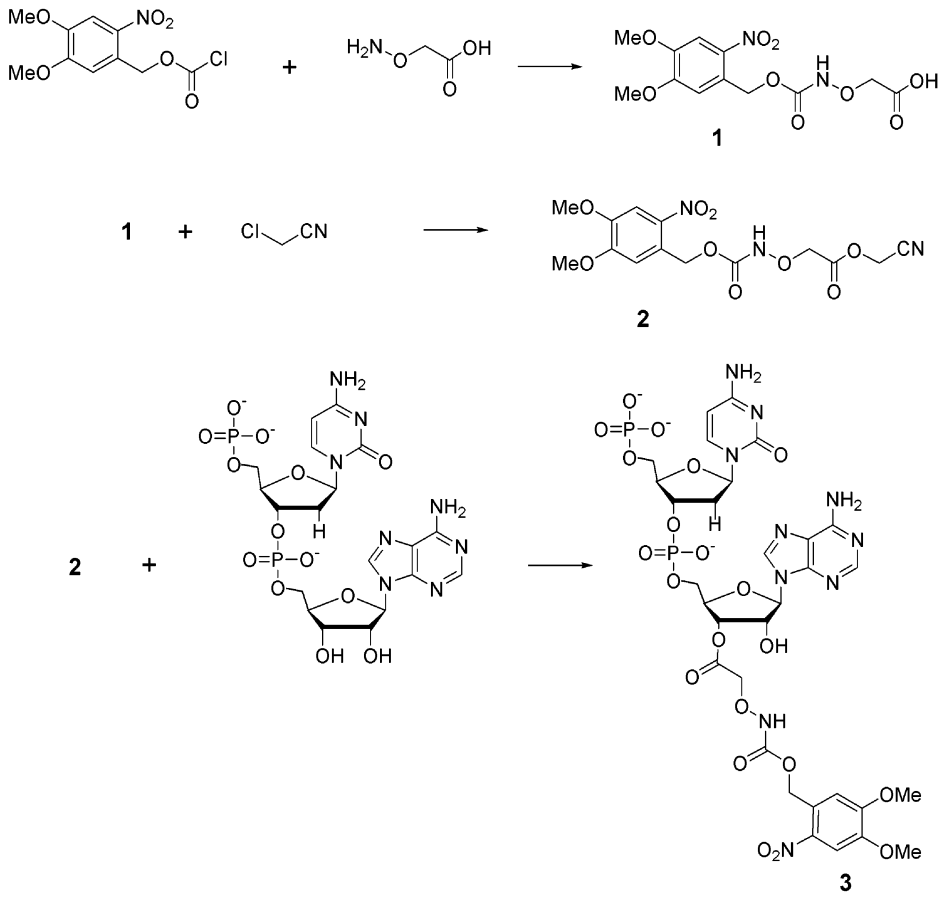
Synthesis of NVOC-aminoacyl-tRNAs. An incubation mixture containing 0.5 A_{260} unit (20 nmol) of an NVOC-aminoacyl-pdCpA and 5 μg of a $tRNA_{CUA}$ transcript lacking the 3′-terminal pCpA moiety (*10*) in 50 μL of 50 mM Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 0.75 mM ATP, 20% DMSO (v/v), and 100 units of T4 RNA ligase was incubated at 37 °C for 60 min. The incubation mixture was treated with 3 M NaOAc, pH 5.3, to a final salt concentration of 0.3 M and then with 2.5 volumes of cold ethanol. The precipitated NVOC-aminoacyl-tRNA was collected by centrifugation. The pellet was washed with ethanol, dried, and then dissolved in H_2O to a final concentration of 1 μg/μL. Analysis was carried out by acid gel electrophoresis (*51*) using RNase-free 8% acrylamide gel in the presence of 100 mM NaOAc, pH 5.0, and 7 M urea. Gels were prerun to warm (∼40 °C), and before loading, samples were heat denatured in the presence of 80% formamide, which was the gel loading solution.

Deprotection of NVOC-Protected Aminoacyl-tRNA. Deprotection was accomplished by irradiation of a cold (2−2.5 °C) aqueous solution of NVOC-protected aminoacyl-tRNAs at a concentration of 1 μg/μL in 1 mM KOAc, pH 4.5, with a 500 W mercury−xenon lamp for 2 min using Pyrex and water filters.

Construction of Expression Plasmids. The gene for rat DNA polymerase β was excised from the plasmid JMpβ5 following site-directed mutagenesis to create a *Nde*I restriction site at the start of the gene. The gene was subcloned into pET28b(+) from the *Nde*I to *Sal*I restriction sites. This vector includes a region coding for a hexahistidine fusion peptide at the N-terminus of the enzyme. Site-directed mutagenesis was then employed to add an amber stop codon to the gene at position 72, which normally codes for lysine.

Translation in a Rabbit Reticulocyte Lysate Protein Biosynthesizing System. To 17.5 μL of nuclease-treated rabbit reticulocyte lysate were added 0.5 μL of an amino acid mixture (1 mM) minus methionine, 1 μL of ^{35}S-labeled

Scheme 1: Synthesis of *N*-(6-Nitroveratryloxycarbonyl)(aminooxy)acetyl-pdCpA[a]



[a] (Aminooxy)acetic acid was protected with 6-nitroveratryl chloroformate and treated with chloroacetonitrile. Finally, the activated ester was coupled to pdCpA, resulting in a 2′,3′ isomeric mixture of *N*-(6-nitroveratryloxycarbonyl)(aminooxy)acetyl-pdCpAs (**3**)

methionine (1000 Ci/mmol, 10 μCi/μL), 0.5 μL of RNasin ribonuclease inhibitor, and 1 μg of an RNA substrate in DEPC-treated water. Following incubation at 30 °C for 90 min, the incubation mixture was analyzed by 20% SDS−PAGE and visualized by autoradiography. When an unnatural amino acid was incorporated, 2.5 μg of the appropriate aminoacyl-tRNA$_{CUA}$ was added to the incubation mixture.

Zinc/Acetic Acid Reduction of the (Aminooxy)acetic Acid N−O Bond in a Modified DNA Polymerase β. Following the incorporation of (aminooxy)acetic acid into position 72, the modified enzyme was purified by Ni-NTA agarose affinity chromatography and was subjected to reductive cleavage. Reductive cleavage of the N−O bond was performed using zinc and aqueous acetic acid. The modified DNA polymerase β containing (aminooxy)acetic acid (22 ng) was subjected to the action of zinc dust (1 mg) in 30 μL of 10% acetic acid for 2 h at 25 °C. Bubbling in the reaction was evident. The peptide fragments were recovered from the surface of the Zn by overnight digestion in the presence of 1 M HCl (60 μL total volume) at 25 °C. The clear solution was treated with 10 volumes of cold acetone followed by centrifugation. The pellet was resuspended in loading buffer and run on a 20% SDS−PAGE gel for 4 h at 5 W.

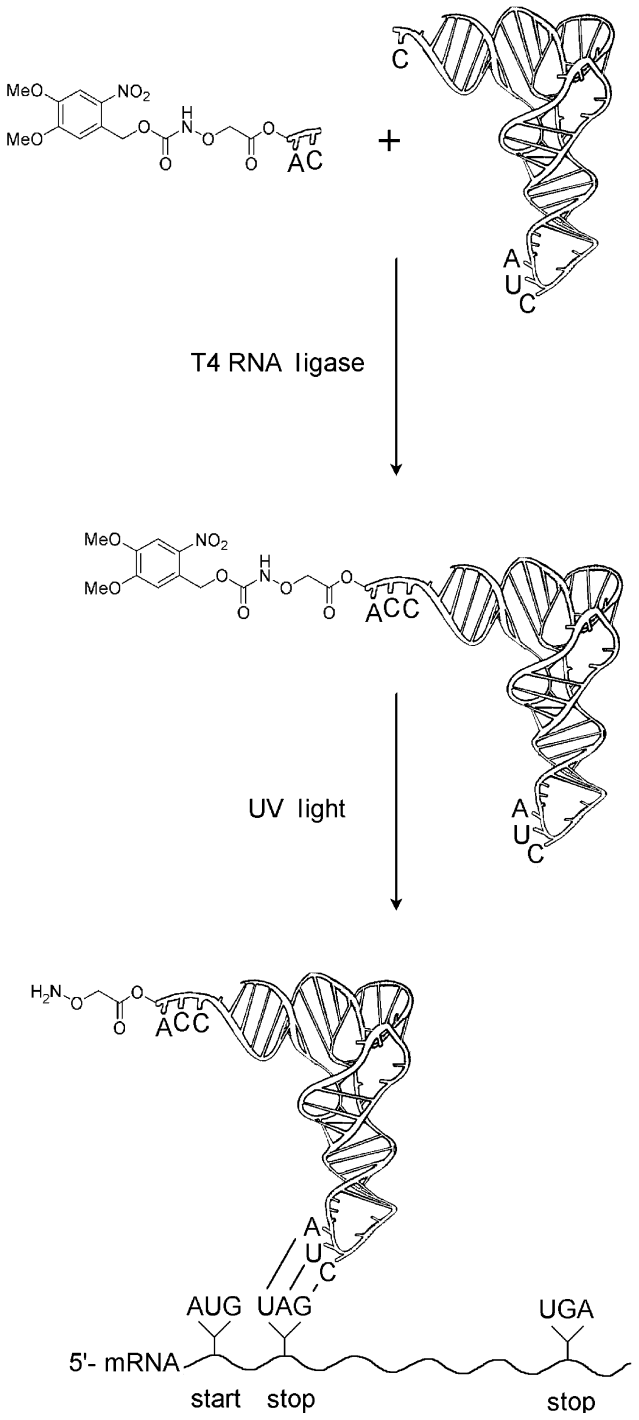
RESULTS

Synthesis of the (aminooxy)acetyl-pdCpA derivative required for preparation of the misacylated suppressor tRNA was accomplished as outlined in Scheme 1. Following N-protection of (aminooxy)acetic acid as the respective nitroveratryloxycarbonyl (NVOC) derivative **1**, the carboxylate moiety was converted to the respective cyanomethyl ester (**2**). Incubation with the tetrabutylammonium salt of pdCpA in dimethylformamide then afforded the desired acylated pdCpA derivative **3**.

Acylated dinucleotide **3** was then ligated to the 3′-end of a suppressor tRNA transcript lacking pCpA at its 3′-terminus (Scheme 2) (*4*−*6*, *10*). Ligation of the aminoacylated dinucleotide to tRNA-C$_{OH}$ was mediated by T4 RNA ligase; the course of the transformation was analyzed by acidic, denaturing PAGE (*51*). The aminoacylated tRNA$_{CUA}$s migrated a slightly lesser distance than did tRNA-C$_{OH}$ (Figure 1).

The enzyme *Escherichia coli* dihydrofolate reductase (DHFR, EC 1.5.1.3) was used in this study for the attempted incorporation of (aminooxy)acetic acid, because it is small (20 kDa), soluble in water, and has been characterized in detail (including X-ray crystallographic analysis) (*52*−*60*). It is also translated in good yields in in vitro protein synthesizing systems (*22*). For the present study, two positions were chosen for modification. On the basis of X-ray crystallographic analysis, it is believed that position 10 exists in a 3_{10}-helical region and on the surface of the enzyme. Position 27 exists in an α-helical region and is buried within the enzyme tertiary structure (*57*). Both positions have previously been used as sites for the incorporation of modified amino acids (*22*, *23*).

Scheme 2: Abbreviated Yeast $tRNA_{CUA}$ Ligation to a 2′,3′ Isomeric Mixture of *N*-(6-nitroveratryloxycarbonyl)(aminooxy)acetyl-pdCpAs and Subsequent Deprotection with UV Light for Use in Suppression Experiments[a]



[a] In this scheme and Figure 1, the deoxycytidine in the penultimate position from the 3′-end of the tRNA is written as C rather than dC in the interest of simplicity.

DHFR was synthesized in vitro using a rabbit reticulocyte lysate biosynthesizing system. Translation was initiated at an AUG codon in the mRNA, coding for a methionine. There are three methionine codons in the 5′-domain of this mRNA. These gave rise to three protein products as shown by SDS−PAGE (Figure 2) and discussed previously (*23*). The protein resulting from the initiation at codon position 1 is full-length

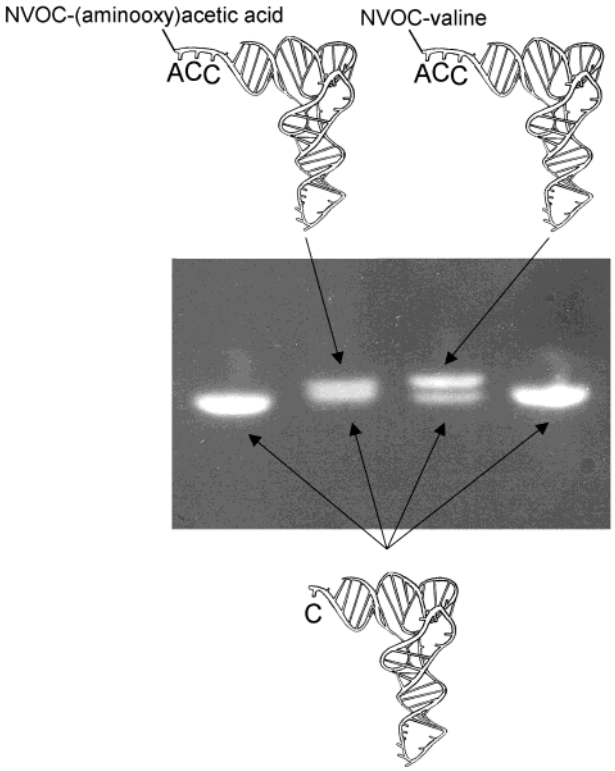


FIGURE 1: The extent of ligation to afford the aminoacyl-tRNAs was analyzed by acid−PAGE [8% acrylamide gel in the presence of 7 M urea and 50 mM sodium acetate, pH 5.0 (*51*)]. The starting material, $tRNA\text{-}C_{OH}$, migrated farther than the aminoacylated analogue. Also, NVOC-(aminooxy)acetyl-tRNA migrated slightly farther than NVOC-valyl-tRNA.

E. coli DHFR but was not the desired product. The desired protein was one that resulted from initiation of translation at position −9 and included a hexahistidine fusion peptide (MIHHHHHHE), which permitted protein purification by affinity chromatography on a nickel nitrilotriacetic acid (Ni-NTA) column (Figure 3) (*61*). The elaborated DHFR proteins lacking the hexahistidine fusion peptide did not bind to the column and were eluted in the wash fractions. After elution of the column with imidazole, only the full-length proteins containing the fusion peptide were observed for both wild-type DHFR (lane 6) and the analogue containing (aminooxy)-acetic acid at position 10 (lane 5).

The suppression efficiency for the incorporation of (aminooxy)acetic acid into position 10 of DHFR was generally 9−15% above background (e.g., see Figure 4). The background value was obtained in the presence of NVOC-protected (aminooxy)acetyl-$tRNA_{CUA}$, apparently by competition from other aminoacyl-tRNAs (*62*). The extent of readthrough in the absence of misacylated suppressor tRNA was shown to be mRNA concentration dependent; an mRNA concentration of 6 μg/mL was utilized to minimize background readthrough (Figure 4). In comparison, the yield of incorporation of (aminooxy)acetic acid into DHFR at position 27 was always lower; the net incorporation above background for the experiment shown in Figure 1 of the Supporting Information was only 3% (13% vs 10% in lanes 1 and 2, respectively). The formation of full-length DHFR in the presence of NVOC-protected (aminooxy)acetyl-$tRNA_{CUA}$ must have been due either to adventitious deprotection in situ or else to competition from other aminoacyl-tRNAs as noted previously (*62*).

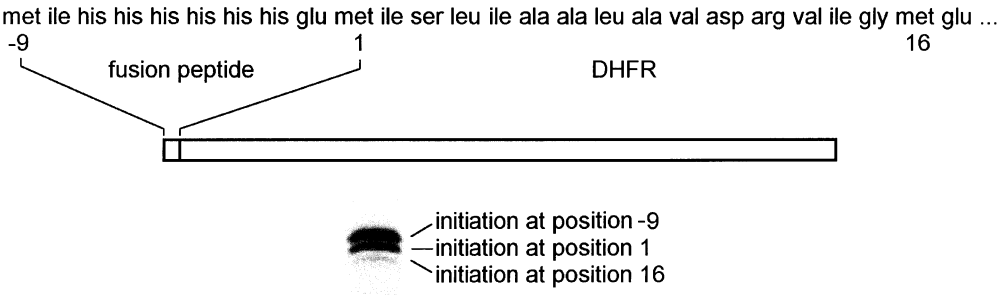


FIGURE 2: In vitro synthesis of *E. coli* DHFR. Three methionine codons are present in the 5′-region of the mRNA for DHFR, and each was capable of initiating translation. The full-length product initiated at position −9 contained the hexahistidine fusion peptide. The other two methionine codons initiated translation less efficiently and produced the two shorter protein fragments, as shown on a typical SDS−PAGE autoradiogram.

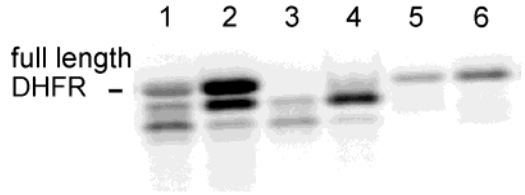


FIGURE 3: Purification of DHFRs by Ni-NTA affinity chromatography. Lanes 1 and 2 contained crude DHFR having (aminooxy)acetic acid and valine at position 10, respectively. Lanes 3 and 4 show wash fractions that did not bind to the column. Lanes 5 and 6 show the full-length proteins containing the fusion peptide and incorporating (aminooxy)acetic acid and valine, respectively, which were retained on the Ni-NTA affinity column.

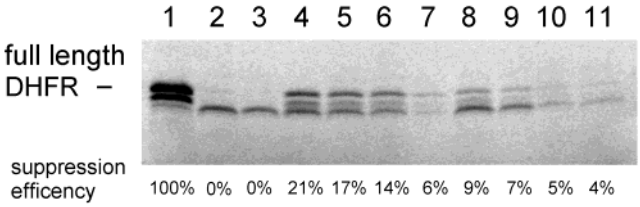


FIGURE 4: Messenger RNA dependence of the synthesis of DHFR. Lane 1 contained 6 μg/mL wild-type mRNA in the absence of any suppressor tRNA. Lanes 2 and 3 contained 6 μg/mL mRNA having UAG at position 10 in the absence of suppressor tRNA. Lanes 4−7 contained 23, 12, 6, and 3 μg/mL DHFR mRNA and deprotected (aminooxy)acetyl-tRNA$_{CUA}$. Lanes 8−11 contained 23, 12, 6, and 3 μg/mL mRNA and NVOC-protected (aminooxy)acetyl-tRNA$_{CUA}$. For incorporation of (aminooxy)acetic acid into position 10 of DHFR, the mRNA concentration giving the best yield of protein with limited amounts of nonspecific readthrough was 6 μg/mL, as shown by lanes 6 and 10. Suppression efficiency was defined as the percentage of full-length DHFR formed by readthrough of a UAG codon, relative to the amount of full-length DHFR produced using wild-type mRNA.

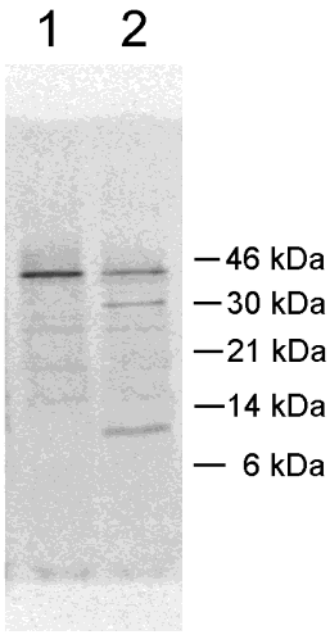


FIGURE 5: Cleavage of (aminooxy)acetic acid at position 72 of DNA polymerase β by Zn dust. Lanes 1 and 2 are polymerase β containing (aminooxy)acetic acid in position 72 without and with treatment with Zn, respectively. Cleavage proceeded to the extent of 54%.

To study the cleavage of a protein containing (aminooxy)acetic acid, a stop codon was introduced into a more central position of a gene, such that cleavage of the resulting protein would afford fragments amenable to analysis by PAGE. Position 72 of DNA polymerase β was used for this purpose.

The gene for rat DNA polymerase β was subcloned into the expression vector pET28b(+). Site-directed mutagenesis was then employed to add an amber stop codon to the gene at position 72, which normally codes for lysine. The incorporation of (aminooxy)acetic acid into rat RNA polymerase β at position 72 was carried out successfully in a rabbit reticulocyte lysate biosynthesizing system, albeit only in 4% yield.

As shown in Figure 5, treatment of the modified polymerase β containing (aminooxy)acetic acid with Zn in aqueous acetic acid effected cleavage of the protein at the N−O bond to afford fragments having the expected molecular masses of 30.6 and 9.9 kDa. To establish the nature of the cleavage reaction, the model peptide *N*-carbobenzyloxyglycyl(aminooxy)acetylalanine methyl ester was treated analogously with Zn in aqueous acetic acid. The formation of the N−O bond cleavage product *N*-carbobenzyloxyglycinamide was verified by C_{18} reversed-phase HPLC in comparison with an authentic sample of the product (data not shown).

DISCUSSION

Although there have been numerous studies involving the use of misacylated tRNAs to incorporate unnatural amino acids into proteins via in vitro protein biosynthesis, virtually all of these have involved tRNAs activated with α-amino acids. The first example of ribosomally mediated formation of a polypeptide analogue of altered connectivity involved the use of a tRNA activated with phenyllactic acid (**4**, Figure 6) (*11*, *37*, *63*); additional examples (**5**−**7**) of depsipeptide formation have been reported more recently (*38*, *64*). These observations demonstrate that the presence of an α-amino group in the aminoacyl-tRNA in the ribosomal A-site is not

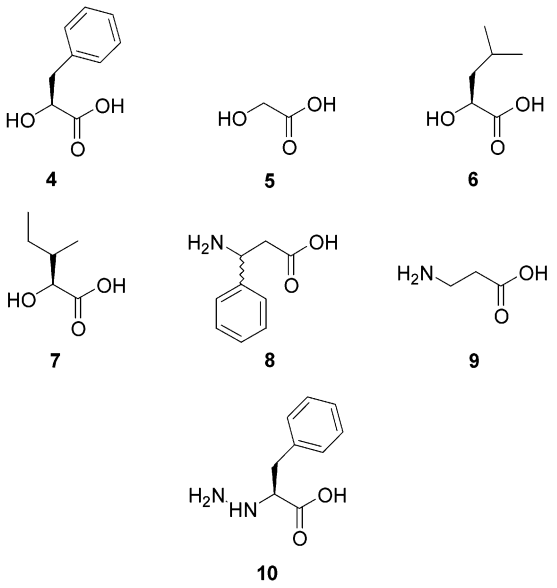


FIGURE 6: Structures of non-α-amino acid analogues successfully incorporated into proteins.

essential for supporting mRNA translation. This indicates that the formation of normal amide bonds is not required to drive polypeptide synthesis, at least if it is absent only occasionally.

A more surprising finding was the ribosome-mediated nucleophilic displacement of chloride ion from *N*-chloroacetylphenylalanyl-tRNA^{Phe} when this species was bound to the ribosomal P-site and phenylalanyl-tRNA^{Phe} was present in the A-site (*65*). In addition to representing an N-alkylation reaction, rather than the usual ribosome-mediated N-acylation, the bond-forming reaction involved atoms positioned quite differently than those which usually participate in peptide bond formation.

Given that the cellular protein biosynthetic machinery is highly evolved for the utilization of α-amino acids having the *S*-configuration, it was also surprising that both isomers of *β*-phenylalanine (**8**) (Figure 6) functioned more efficiently than *S*-phenylalanine as donors in the peptidyltransferase reaction (*7*). Although less efficient than phenylalanine as an acceptor in the peptidyltransferase reaction, *β*-phenylalanine nonetheless formed a peptide bond in 8% yield (*8*). Likewise, *β*-alanine (**9**) (Figure 6) was incorporated into a hexadecapeptide in 9% yield (*63*) and into T4 lysozyme in 5% yield (*14*). More recently, Killian et al. (*39*) have demonstrated that α-hydrazinophenylalanine (**10**, Figure 6) can utilize either N^{α} or N^{β} in peptide bond formation and that this amino acid analogue could be incorporated into DHFR at position 10 with 10% suppression efficiency.

These earlier studies suggested that (aminooxy)acetic acid might also be capable of participating in the peptidyltransferase reaction. The observed suppression efficiency of (aminooxy)acetyl-tRNA$_{CUA}$ for readthrough of a UAG codon at position 10 of DHFR mRNA was 9−15% in replicate experiments, consistent with findings for *β*-amino acids and α-hydrazinophenylalanine. The reason for the less efficient incorporation of (aminooxy)acetic acid into position 27 of DHFR is not entirely clear, although other amino acids have also been noted to effect suppression of a UAG codon more efficiently at one position than another within the same mRNA (*22*, *62*, *66*).

In addition to the intrinsic interest in defining the ability of an aminooxy acid to participate in protein biosynthesis, the derived proteins are also of special interest. Peptides containing aminooxy acids have been the subject of several studies in recent years. Experimental (*40*−*42*) and computational (*43*, *44*) studies of peptides containing an aminooxy acid residue are consistent in their findings that the aminooxy acid residue induces conformational rigidity and the formation of a novel 1.8_8 helix, similar to a *γ*-turn, that is independent of the side chain on the aminooxy acid moiety. Due to their resistance to cellular proteases, *β*-amino acids have been of great interest for the synthesis of modified polypeptides (*67*, *68*) and for inclusion in peptidomimetics in the design of potential drugs (*69*). The conformational preferences of polyamides incorporating *β*-amino acids have been studied (*70*). The use of aminooxy acids, which form peptide analogues having the same number of backbone atoms as *β*-amino acids, has the potential to impart conformational rigidity (*40*−*44*, *71*) which may confer substantial advantage in drug design. It may be noted that an aminooxy acetic residue has also been incorporated into the side chain of a modified polypeptide as a linker residue (*72*).

Assuming that this conformational rigidity is also present in proteins containing aminooxy acids at one or more specific positions, one can envision their utility in creating protein analogues, e.g., in which thermal stability has been increased by replacing essential *γ*-turns with an appropriate aminooxy acid. The use of aminooxy acids at specific positions to probe structural requirements for local turn motifs or conformational flexibility in support of protein function can also be envisioned.

A second reason for interest in proteins containing aminooxy acids at predetermined sites relates to the N−O bond present in such species. This functionality can be cleaved chemically by a number of reagents (*45*−*49*), and it has now been verified that the N−O bond at the site of incorporation of (aminoxy)acetic acid into a protein is amenable to specific cleavage. This property can be used to good advantage for site-specific protein cleavage, as has recently been demonstrated for proteins containing allylglycine (*73*−*75*).

ACKNOWLEDGMENT

We thank Dr. Vladimir Karginov for the plasmid containing the hexahistidine-linked dihydrofolate reductase gene, Dr. Sergey Mamaev for the plasmid from which abbreviated yeast tRNA$^{Phe}_{CUA}$ was transcribed, and Dr. Bixun Wang for initially testing the feasibility of (aminooxy)acetic acid as a ribosomal substrate.

SUPPORTING INFORMATION AVAILABLE

One figure showing the incorporation of (aminooxy)acetic acid into position 27 of DHFR. This material is available free of charge via the Internet at http://pubs.acs.org.

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