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Genetic Incorporation of a Phenanthroline-Containing Amino Acid in *Escherichia coli*

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A simple and general method that selectively introduces metal binding sites into a protein can greatly increase the ability to design and biosynthesize artificial metalloproteins. Here, we report the incorporation of a phenanthroline-containing amino acid (Phen-Ala) into proteins in *Escherichia coli* by using the $tRNA^{Tyr}_{CUA}$ and tyrosyl aminoacyl-tRNA synthetase pair (BpyRS) from *Methanococcus jannaschii*, which was originally developed for a bipyridine-containing amino acid (Bpy-Ala). The incorporation efficiency of BpyRS for Phen-Ala was comparable to that for Bpy-Ala. Because of its high metal-binding ability and characteristic spectral properties, Phen-Ala can be a useful alternative to the existing metal-chelating amino acids for the design and synthesis of artificial metalloproteins.

Key Words : Phenanthroline, Metal-chelating amino acids, Proteins

Introduction

Approximately one-third of all proteins are estimated to require metal ions to perform their biochemical functions. These metal ions are responsible for important biological processes, including photosynthesis, biomolecular recognition, synthesis and degradation of biomolecules, and various redox reactions. Because metal ion-containing proteins (metalloproteins) play such critical roles, a number of studies have been conducted to understand their structure and function. The resulting knowledge has helped us in rationally designing artificial metalloproteins with interesting structures, functions and catalytic activities.[1,2] Current approaches for the introduction of metal binding sites into a protein include conjugation of a metal binding group to an existing protein binder,[3] covalent modification of a protein with a metal binding group,[4,5] and de novo protein design.[6,7] Although many interesting applications of the proteins generated through these techniques have been reported, introduction of metal binding sites into proteins remains a challenging process. Therefore, a simple, general method for selective introduction of a metal binding site into proteins would be highly beneficial for engineering catalytic and redox active sites, radioisotope-binding sites, spectroscopic probes, and structural elements in proteins.

Recently, a general method that has been developed to genetically encode unnatural amino acids (UAAs) has been applied to biosynthesize mutant proteins containing unnatural functional groups.[8,9] By using this method, two metal-chelating amino acids, bipyridylalanine (Bpy-Ala) and 8-hydroxyquinolinylalanine (HQ-Ala), were incorporated into proteins with high efficiency and selectivity (Figure 1).[10,11] These amino acids site-specifically created a metal binding site in the proteins and introduced novel functions such as sequence-specific DNA-cleavage,[12] enhanced phasing power for crystallographic structure determination,[11] and affinity tags for protein purification.[13] Although these metal chelating amino acids have great potential for the use in the biosynthesis of metallproteins, development of more chelating groups is necessary for the biosynthesis of proteins with more interesting and varied applications. Hence, 1,10-phenanthroline (Phen) – one of the most extensively used metal-chelating groups in a wide range of chemical and biochemical experiments – was considered as a new metal chelating group.[5,14,15] Phen has been introduced into proteins through chemical modification to generate metal binding sites, and these proteins have been used as metalloenzymes and DNA-cleaving proteins.[5,14] Therefore, it was expected that genetic incorporation of a Phen-containing amino acid into proteins would facilitate the design and biosynthesis of metalloproteins.

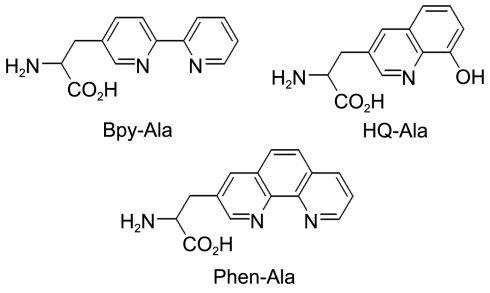


Figure 1. The structures of metal-chelating amino acids.

Experimental

General Method. All chemicals and DNA oligomers were obtained from commercial sources and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Varian Inova-500 (500 MHz for ^{1}H, and 125 MHz for ^{13}C) spectrometer with chemical shifts recorded relative to tetramethylsilane.

Synthesis of Ethyl 4-Chloro-1,10-phenanthroline-3-carboxylate (2). Ethyl 4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate[16] (**1**, 5.00 g, 18.6 mmol) was dissolved in $POCl_3$ (25 mL) and the solution was refluxed for 30 min. The reaction mixture was cooled to room temperature, and 25% ethyl acetate in diethyl ether (100 mL) was added to the mixture. The solid was filtered and washed with 25% ethyl acetate in diethyl ether. The crystalline solid was suspended in ethyl acetate (100 mL) and saturated aqueous sodium bicarbonate solution (100 mL) was added. The mixture was stirred for 10 min and the organic layer was dried over sodium sulfate. The solution was concentrated under reduced pressure to give the product (3.69 g, 69%) as a brownish solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.50 (t, 3H), 4.55 (q, 2H), 7.75 (dd, 1H), 7.98 (d, 1H), 8.33 (dd, 1H), 8.43 (d, 1H), 9.29 (s, 1H), 9.47 (s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.4, 62.5, 122.9, 124.4, 125.4, 126.9, 128.5, 129.5, 136.5, 143.1, 145.4, 148.1, 150.4, 151.2, 164.6. LC-MS (ESI) calcd for $C_{15}H_{11}N_2O_2$ (M+H) 287.71, obsd. 287.20.

Synthesis of (4-Chloro-1,10-phenanthrolin-3-yl)methanol (3). To a stirred suspension of compound **2** (2.86 g, 10.0 mmol) in MeOH was added sodium borohydride (1.50 g, 40 mmol), and the mixture was stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure, dissolved in ethyl acetate and washed once with saturated aqueous sodium bicarbonate solution. The organic phase was dried over sodium sulfate and concentrated under reduced pressure to give a crude product which was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2) to afford the product (1.49 g, 61%) as a yellowish powder. ^{1}H-NMR (500 MHz, CD_3OD) δ 5.02 (s, 2H), 7.79 (dd, 1H), 8.04 (d, 1H), 8.29 (d, 1H), 8.47 (dd, 1H), 9.10 (dd, 1H), 9.18 (s, 1H). ^{13}C-NMR (125 MHz, CD_3OD) δ 59.8, 121.8, 124.0, 126.3, 128.0, 128.9, 134.4, 136.9, 140.5, 145.1, 145.6, 149.2, 150.3. LC-MS (ESI) calcd for $C_{13}H_9N_2OCl$ (M+Na) 267.68, obsd. 267.04.

Synthesis of (1,10-Phenanthrolin-3-yl)methanol (4). To a stirred solution of compound **3** (2.3 g, 9.4 mmol) in methanol (100 mL), Pd/C (0.12 g) and triethylamine (13 mL, 94 mmol) were added, and the mixture was stirred under hydrogen (balloon) for 48 h at room temperature. The reaction mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure to yield a yellowish solid. The crude product was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2) to afford the product (1.09 g, 55%) as a yellowish powder. ^{1}H-NMR (500 MHz, CD_3OD) δ 4.88 (d, 2H), 7.68 (qd, 1H), 7.80 (d, 2H), 8.28 (d, 1H), 8.34 (dt, 1H), 9.02 (m, 2H). ^{13}C-NMR (125 MHz, CD_3OD) δ 61.5, 123.3, 126.6, 126.7, 128.7, 128.9, 134.1, 136.6, 137.3, 144.5, 145.4, 149.2, 149.7. LC-MS (ESI) calcd for $C_{13}H_{10}N_2O$ (M+H) 211.23, obsd. 211.15.

Synthesis of Diethyl 2-((1,10-Phenanthrolin-3-yl)methyl)-2-acetamidomalonate (5). Compound **4** (191 mg, 0.91 mmol) was dissolved in methylene chloride (5 mL), and thionyl chloride (0.33 mL, 4.5 mmol) was added. The mixture was stirred for 1 h at room temperature. The reaction mixture was washed with saturated aqueous sodium bicarbonate solution, dried over magnesium sulfate, and concentrated under reduced pressure to give a brownish solid (210 mg) which was directly used for the next step without further purification. A mixture of diethyl acetamidomalonate (220 mg, 1.0 mmol) and sodium hydride (40 mg, 1.0 mmol, 60% in mineral oil) in dry DMF (5 mL) was stirred for 30 min at 0 °C. To the solution was added the crude product in dry DMF (2.0 mL) at 0 °C, and the mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc (30 mL) and washed with 10% aqueous sodium thiosulfate solution (2 × 30 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2) to give the product (240 mg, 65%) as a white solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.09 (d, 5H), 3.97 (s, 2H), 4.30 (m, 8H), 5.17 (d, 1H), 6.61 (s, 1H), 7.65 (dd, 1H), 7.45 (d, 1H), 7.81 (d, 1H), 7.91 (d, 1H), 8.26 (d, 1H), 8.86 (d, 1H), 9.19 (d, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ 35.4, 56.6, 62.8, 63.2, 67.3, 123.3, 126.3, 127.1, 128.4, 128.7, 130.7, 136.2, 137.0, 145.6, 146.3, 150.6, 151.8, 166.6, 167.3, 169.7, 169.9. LC-MS (ESI) calcd for $C_{22}H_{23}N_3O_5$ (M+H) 410.44, obsd. 410.30.

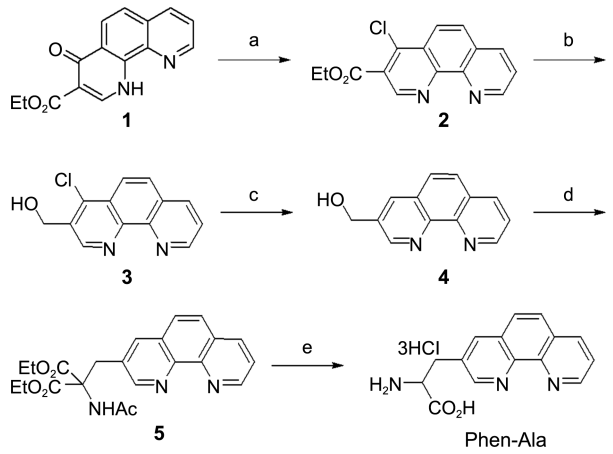
Synthesis of Phen-Ala. Compound **5** (205 mg, 0.50 mmol) in 12 M HCl was heated to reflux for 6 h. The reaction mixture was concentrated under reduced pressure to give the product (186 mg, 99%) as a yellowish powder in a HCl salt form. ^{1}H-NMR (500 MHz, D_2O) δ 3.49 (t, 2H), 3.71 (s, 1H), 4.30 (t, 1H), 8.04 (s, 2H), 8.09 (dd, 1H), 8.47 (s, 1H), 8.96 (s, 1H), 8.97 (d, 1H), 9.04 (d, 1H). ^{13}C-NMR (125 MHz, D_2O) δ 33.5, 40.2, 54.3, 124.9, 126.6, 129.1, 129.9, 133.6, 136.9, 139.3, 143.9, 145.6, 151.0, 170.2, 171.7. LC-MS (ESI) calcd for $C_{15}H_{14}N_3O_2$ (M+H) 268.10, obsd. 268.13.

Chloramphenicol Resistance Assay. Bacterial cells (DH10B) containing pBK-BpyRS and pRep/YC[17] were cultured on LB-agar plates containing kanamycin (50 μg/mL), tetracycline (12 μg/mL) and chloramphenicol (20 μg/mL) in the presence of Bpy-Ala (0.6 mM), Phen-Ala (0.6 mM) and in the absence of UAA, respectively. The plates were incubated at 37 °C for 2448 hours.

Protein Expression and Purification. Site-directed mutagenesis was used to introduce the GFP221TAG mutation into the GFP gene in the plasmid, pBAD-GFP. The plasmid containing the amber mutation (pBAD-GFP211TAG) was co-transformed with pSup-BpyRS[12] into *E. coli* DH10B. Cells were amplified in LB supplemented with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL). Starter culture (2.5 mL) was used to inoculate 100 mL LB supplemented with ampicillin (100 μg/mL) and 1 mM Phen-Ala or Bpy-Ala at 37 °C, and expression was induced at OD 0.8 (600 nm) by adding 0.2% L-arabinose. Cells were grown at 37 °C for overnight, harvested by centrifugation, and frozen at -80 °C. Purification by the Ni-NTA resin was performed under native conditions according to the manufacturer's protocol.

Results and Discussion

To incorporate a Phen-containing amino acid into proteins,



Scheme 1. Synthesis of Phen-Ala
Conditions: (a) $POCl_3$, reflux, 0.5 h, 69%; (b) $NaBH_4$, MeOH, room temperature, 4 h, 61%; (c) H_2, Pd/C, Et_3N, MeOH, room temperature, 48 h, 55%; (d) $SOCl_2$, CH_2Cl_2, room temperature, 1 h and then, NaH, diethyl acetamidomalonate, DMF, room temperature, 1 h, 65%; (e) 12 N HCl, reflux, 6 h, 99%.

Phen-Ala (Figure 1) was designed and synthesized. The synthesis of Phen-Ala started with ethyl 4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate[16] (**1**) and was completed in 5 steps in 15% overall yield. The starting material was refluxed in phosphoryl chloride to produce compound **2**. The ester group was reduced by sodium borohydride, and the chlorine in the Phen ring was replaced with hydrogen by Pd-catalyzed hydrogenolysis to produce compound **4**. Thionyl chloride was used to replace the hydroxyl group with a chlorine atom, and ethyl acetamidomalonate was introduced through an alkylation reaction using sodium hydride as a base. Subsequent acidic decarboxylation and hydrolysis afforded Phen-Ala in its racemic form (Scheme 1). For incorporation of Phen-Ala in *E. coli*, the aminoacyl tRNA synthetase (BpyRS) developed for Bpy-Ala from the tyrosyl-synthetase of *Methanococcus jannaschii* was initially tested. The crystal structure of BpyRS complexed with Bpy-Ala showed that the amino acid binding site could accommodate the extra two carbons of Phen-Ala (Figure 2).[10] The structure

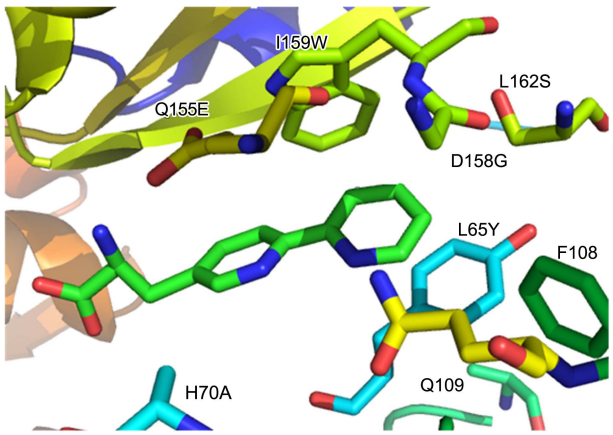


Figure 2. The X-ray crystal structure of BpyRS complexed with Bpy-Ala (PDB ID: 2PXH).[10]

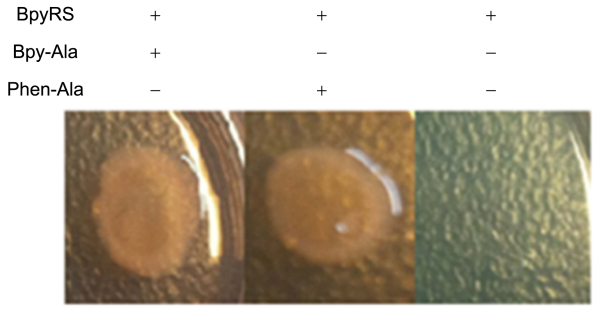


Figure 3. Chloramphenicol resistance assay. Bacterial cells containing pBK-BpyRS and pRep/YC (Asp112TAG on cat gene) were cultured on agar plates containing chloramphenicol (20 μM) and the indicated amino acid (0.6 mM).

also showed that the two pyridine rings in Bpy-Ala are not on the same plan; one of the rings was twisted at 21 degree, while the Phen ring has a planar structure. However, we expected that BpyRS might still recognize and aminoacylate Phen-Ala to the corresponding tRNA.

To test whether BpyRS could catalyze the aminoacylation of Phen-Ala, the amber stop codon (Asp112TAG) in the *cat* gene was suppressed in the presence and absence of Phen-

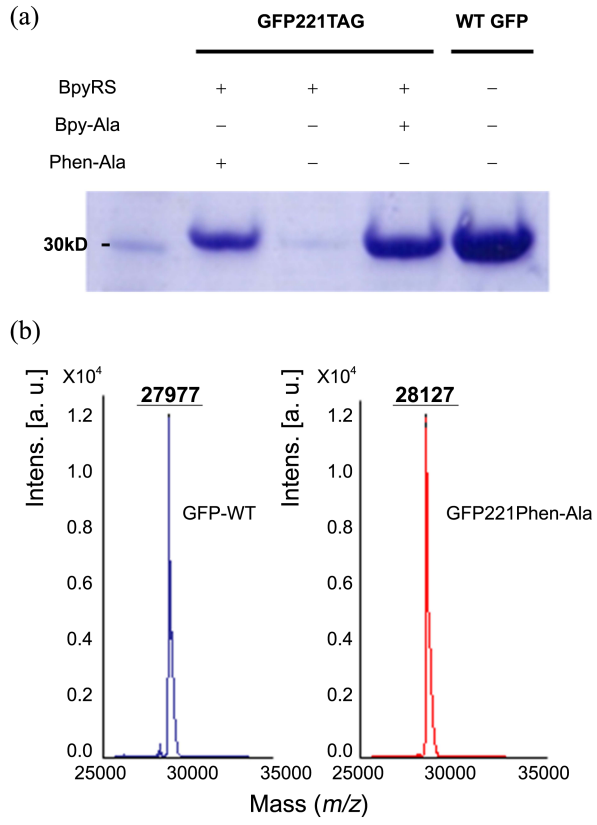


Figure 4. Expression of GFP gene with an amber codon at position 221 (Leu) in the presence of the *Mj*tRNA$^{Tyr}_{CUA}$/BpyRS. (a) SDS-PAGE analysis of the mutant GFP. (b) MALDI-TOF mass spectrometry results for GFP-WT (left) and GFP221Phen-Ala (right). GFP-WT, observed 27977; GFP221Phen-Ala, observed 28127; theoretical mass difference between leucine and Phen-Ala, 136; observed mass difference between GFP-WT and GFP221Phen-Ala, 150.

Ala (1 mM).[17] Bacterial cells expressing the tRNA/BpyRS pair and the mutant *cat* gene were cultured on agar plates containing chloramphenicol (20 μM). The cells grew only on the plate containing Phen-Ala (0.6 mM), and no cell growth was observed in the absence of the amino acid (Figure 3). This result showed that BpyRS recognizes Phen-Ala and catalyzes its aminoacylation to the corresponding tRNA.

To further examine the efficiency of BpyRS to incorporate Phen-Ala into proteins, the green fluorescent protein (GFP) gene with an amber codon at position 221 was expressed in *E. coli* in the presence of BpyRS and the corresponding tRNA. Culturing in Lysogeny Broth (LB) yielded 9.6 mg/L of full-length protein in the presence of 1 mM Phen-Ala, whereas little full-length protein was detected by SDS-PAGE analysis in the absence of the amino acid (Figure 4(a)). Although the same expression with 1 mM Bpy-Ala afforded a slightly better yield (13.5 mg/L), the efficiency of BpyRS for Phen-Ala was observed to be good enough to provide full-length proteins in sufficient amounts for biochemical applications. MALDI-TOF mass spectrometric analysis of the purified protein confirmed the incorporation of Phen-Ala and the fidelity of BpyRS (Figure 4(b)).

Conclusion

In summary, a phenanthroline-containing amino acid (Phen-Ala) was incorporated into proteins in *E. coli* by using the *Mj*tRNA$^{Tyr}_{CUA}$/BpyRS pair, which was originally developed for the incorporation of Bpy-Ala. The incorporation efficiency of BpyRS for Phen-Ala, which was evaluated by incorporating the amino acid into GFP, was found to be comparable to that for Bpy-Ala. Although other metal-chelating amino acids such as Bpy-Ala and HQ-Ala have been successfully incorporated into proteins, Phen-Ala can be a useful alternative to these amino acids owing to its high metal-binding ability and characteristic spectral properties.

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