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In Cellulo Synthesis of Proteins Containing a Fluorescent Oxazole Amino Acid

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Abstract

Genetic code expansion has enabled many noncanonical amino acids to be incorporated into proteins *in vitro* and *in cellulo*. These have largely involved α -L-amino acids, reflecting the substrate specificity of natural aminoacyl-tRNA synthetases and ribosomes. Recently, modified *E. coli* ribosomes, selected using a dipeptidylpur-omycin analogue, were employed to incorporate dipeptides and dipeptidomimetics. Presently, we report the *in cellulo* incorporation of a strongly fluorescent oxazole amino acid (lacking an asymmetric center or α -amino group) by using modified ribosomes and pyrrolysyl-tRNA synthetase (PylRS). Initially, a plasmid encoding the RRM1 domain of putative transcription factor hnRNP LL was cotransformed with plasmid pTECH-Pyl-OP in *E. coli* cells, having modified ribosomes able to incorporate dipeptides. Cell incubation in a medium containing oxazole **2** resulted in the elaboration of RRM1 containing the oxazole. Green fluorescent protein, previously expressed *in vitro* with several different oxazole amino acids at position 66, was also expressed *in cellulo* containing oxazole **2**; the incorporation was verified by mass spectrometry. Finally, oxazole **2** was incorporated into position 13 of MreB, a bacterial homologue of eukaryotic cytoskeletal protein actin F. Modified MreB expressed *in vitro* and *in cellulo* comigrated with wild type. *E. coli* cells expressing the modified MreB were strongly fluorescent and retained the *E. coli* cell rod-like phenotype. For each protein studied, the incorporation of oxazole **2** strongly increased oxazole fluorescence, suggesting its potential utility as a protein tag. These findings also suggest the feasibility of dramatically increasing the repertoire of amino acids that can be genetically encoded for protein incorporation *in cellulo*.

Oxazole amino acids having favorable characteristics as fluorophores¹ can be incorporated into proteins *in vitro* by modified ribosomes.^{2,3} Eleven synthetic oxazole and thiazole amino acids^{3,4} were incorporated into green fluorescent protein (GFP) from activated suppressor tRNA_{CUA}S. The fluorescent GFPs had significant Stokes shifts (30–70 nm) and strongly enhanced fluorescence relative to free oxazoles. GFP containing oxazole **1** (Figure 1) at position 66 within the β -barrel structure was four times brighter than wild-type.^{3–5} Inclusion of oxazole **1** within the peptide tetraglycine tripled its brightness.⁴

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ASSOCIATED CONTENT

Supporting Information

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Experimental procedures for *in vitro* and *in cellulo* protein synthesis and characterization; additional tables and figures (PDF)

The authors declare no competing financial interest.

One important application for fluorescent proteins is as genetically encodable labels; fusion proteins incorporating GFP at one end enable cellular trafficking and macromolecular interactions of the protein to be monitored in real time.⁶ While fluorescent proteins are powerful tools for monitoring protein behavior, there are some limitations. Because GFP fluorophore is formed by the cyclization and oxidation of three contiguous amino acids,^{6b} the nascent translated protein is not initially fluorescent. Some fluorescent proteins are also converted to their fluorescent forms only after a significant delay;^{6b} thus early events cannot be monitored. Some of the formed fluorophores also lack stability, further limiting their utility. Potentially more serious is the size of the fluorescent proteins, which can be larger than the proteins whose behavior is being studied.⁷ The smaller oxazole amino acids and strong fluorescence of proteins containing oxazoles suggests their use as protein labels in lieu of GFP.

Many proteins containing nonproteinogenic amino acids have been expressed *in cellulo*,⁸ but the substrate specificity of natural ribosomes and the requirement for an aminoacyl-tRNA synthetase that can activate amino acid analogues generally limit the incorporation to α -L-amino acids.^{8,9} We anticipated that the ribosomes shown to incorporate oxazoles into proteins *in vitro*^{3,4} would enable their incorporation *in cellulo*. For tRNA_{CUA} activation, we explored the use of PylRS and cognate suppressor tRNA^{Pyl} already used for analogue incorporation, and having a relaxed specificity for an α -amino moiety.¹⁰ We focused on oxazole **2** due to its good quantum yield and longer wavelength emission than oxazole **1** (458 vs 389 nm).

The potential of oxazole **2** to act as a PylRS substrate was evaluated by computational docking using Autodock Vina v.1.1.2;^{11,12} the results were compared with the X-ray crystal structure of the PylRS catalytic domain bound to Pyl and ATP (Figure S1 and Table S1). Oxazole **2** fit easily in the active site cavity and the six key amino acid residues close to the Pyl substrate (Leu305, Tyr306, Leu309, Asn346, Cys348, and Trp417) were in reasonable proximity to oxazole **2**. *E. coli* cells having modified ribosomes that incorporate dipeptidomimetics were prepared by transformation of BL-21(DE-3) with plasmid pUCrrnB010328R4.¹³

The hnRNP LL RRM1 domain was chosen for translation optimization in cells with modified ribosomes because it is small (~14 kDa) and expressed well *in vitro*.^{2c,14} *E. coli* cells having modified ribosomes were cotransformed with pTECH-Pyl-OP and plasmid pETRRM1-24 (i.e., having a TAG codon at position 24). In parallel, cotransformation of cells having only wild-type ribosomes and pETRRM1-24 was effected. The synthesis of RRM1 in the two cultures was run in parallel, in the presence of 2 mM *p*-cyanophenylalanine (CNPhe) (Figure S2). CNPhe was chosen because it is fluorescent and was shown previously to be incorporated into proteins *in cellulo*.^{10c} As seen in Figure S2, cells containing wild-type and modified ribosomes both incorporated CNPhe. In a parallel experiment oxazole amino acid **2** was incorporated into the same position in RRM1; incorporation was dependent upon the PylRS-tRNA^{Pyl} orthogonal pair (Figure S3).

A repeat experiment was done to enable detailed RRM1 characterization (Figure 2). While the fluorescence emission maxima for the two proteins differed (440 nm for RRM1

containing **2** vs 405 nm for RRM1 containing CNPhe), RRM1 containing **2** exhibited ~50-fold greater fluorescence emission intensity (Figure 2B). As observed for structurally related species,^{3,4} oxazole **2** as a constituent of RRM1 emitted at 440 nm (vs 460 nm for free oxazole **2**) and exhibited significantly increased fluorescence.

The analysis of proteins containing oxazole **2** was extended to include green fluorescent protein (GFP), which had demonstrated strongly enhanced fluorescence after incorporation of oxazole **1** *in vitro*.^{3a} Following verification that oxazole **2** can be incorporated into GFP *in vitro* using modified ribosomes (Figure S4),⁴ *in cellulo* synthesis in the presence of oxazole **2** afforded 10-fold less modified GFP than RRM1 (0.4 mg/L vs 4 mg/L, Figure 3A), but the modified GFP exhibited ~2-fold greater fluorescence intensity with very similar emission maxima (425 nm for the modified GFP; 432 nm for RRM1) (Figure 3B). The purified GFP-66-2 produced *in cellulo* was analyzed by mass spectrometry (Figure S5), verifying the presence of oxazole **2**. While GFP containing oxazole **1** at position 66 was ~4 times brighter than wild type,^{3a} the lesser quantum yield of **2** as compared with **1** (0.60 vs 0.90)⁴ suggested the need to verify that GFP containing **2** could be detected readily. Accordingly, a 2 μ M solution of this protein was compared directly with equimolar solutions of GFP and blue fluorescent protein (BFP). As shown (Figure S6), GFP containing **2** was brighter than wild-type GFP; little BFP fluorescence could be detected.¹⁵

The possible use of oxazole **2** as a constituent to visualize bacterial proteins during cell growth was addressed using *E. coli* MreB as a model. MreB is a homologue of actin F, the cytoskeletal element of eukaryotes;¹⁶ its role in prokaryotes has been studied.¹⁷ MreB polymerizes to form filaments similar to actin microfilaments, which are implicated in bacterial processes including cell shape maintenance, chromosome segregation, and motility. However, such studies often involve the use of fluorescent tags (e.g., fusion proteins with GFP, YFP, and RFP). The fusion proteins can be visualized in *E. coli* with good spatial and temporal resolution¹⁸ but can produce artifacts due to the large size of the fluorescent protein labels.¹⁹ Oxazole-labeled proteins may be useful alternatives; their small size should diminish the risk of observing artifacts.

Plasmid pETMreB was used to express MreB *in cellulo*.²⁰ A second plasmid, pETMreB13, was prepared by replacing the Leu13 codon (TTG) with TAG. This position was chosen for oxazole **2** based on structural data for MreB; the N-terminal domain appears not to be involved in self-polymerization or protein binding,²¹ such that introduction of oxazole **2** seemed unlikely to disrupt MreB structure. Both plasmids were studied in an *in vitro* translation system to characterize their efficiency of translation as wild-type and modified proteins (Figure S4). MreB and GFP both afforded good yields of the wild-type proteins. The suppression yield for oxazole **2** at position 13 was 9%, entirely satisfactory for further study *in cellulo*. For the *in cellulo* experiment, both plasmids with MreB genes (wild-type and mutant) were cotransformed with pTECH-Pyl-OP into BL-21 (DE-3)-rrnB010328R4 cells and the level of expression of MreB from the wild-type gene was checked by analysis of the cultured cells before and after cell lysis (Figure S7). Unsurprisingly, only a small amount of protein was present in the soluble fraction, undoubtedly due to the well documented ability of MreB to polymerize and form complexes with other cellular proteins.^{17a,b,d,21,22} Nonetheless, purification of the putative (wild-type and modified) MreB by

Strep-tactin chromatography did afford material with the expected mobility (Figure 4). The presence of the modified protein as a small percentage of wild type (as also observed *in vitro*, Figure S4) suggests that the introduction of oxazole **2** at position 13 did not affect the ability of the modified protein to polymerize *in cellulose*.

The culture of cells producing wild-type and modified MreB for microscopy was carried out using 3 mM oxazole **2** in 2YT medium until OD₆₀₀ < 1.0 was reached. Cells expressing BFP and cells harboring pETMreB grown without oxazole **2** were prepared simultaneously as positive and negative controls for the study of cell fluorescence. The cells were washed five times with sterile PBS solution to remove excess unincorporated oxazole. Microscopy was done with a low resolution fluorescence microscope (Figure 5). The brightest fluorescence was detected for cells harboring the plasmid for modified MreB, grown in the presence of free oxazole **2** (Figure S8). The cells producing fluorescent MreB exhibited a greater fluorescence intensity than cells producing wild-type MreB but grown in the presence of oxazole **2** (and thus potentially containing residual **2** not removed by washing). Since MreB is not anticipated to be present in the bacteria at high concentration, the fluorescence intensity suggests that oxazole **2** incorporated into MreB is significantly brighter than the free oxazole, as noted for all other proteins studied.^{3–5} We do not currently know whether modified MreB expressed *in cellulose* is incorporated into the cytoskeletal structure, the integrity of which is required for normal bacterial cell morphology.^{21,23,24}

However, the *E. coli* containing MreB with oxazole **2** retained their rod-like structure. From the perspective of replacing fluorescent protein labels with oxazole amino acids, it is significant that the fluorescence of the cells expressing MreB containing oxazole **2** was brighter than the fluorescence from the cells expressing BFP (Figures 5 and S8).

The foregoing data support the conclusion that our modified ribosomes can mediate the *in cellulose* incorporation of a heterocyclic amino acid containing neither an α -amine moiety nor any asymmetric center. The ability to express proteins *in cellulose* having an embedded heterocyclic motif is unprecedented and may provide access to cellular macromolecules containing a significantly enhanced repertoire of structural constituents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1) (a). Baranov MS; Lukyanov KA; Ivashkin PE; Yampolsky IV Efficient synthetic approach to fluorescent oxazole-4-carboxylate derivatives. *Synth. Commun.* 2013, 43, 2337–2342. (b) Ferreira PMT; Castanheira EMS; Monteiro LS; Pereira C; Vilaca H A mild high yielding synthesis of oxazole-4-carboxylate derivatives. *Tetrahedron* 2010, 66, 8672–8680.
- (2) (a). Dedkova LM; Fahmi NE; Paul R; del Rosario M; Zhang L; Chen S; Feder G; Hecht SM β -Puromycin selection of modified ribosomes for *in vitro* incorporation of β -amino acids.

- Biochemistry 2012, 51, 401–415. [PubMed: 22145951] (b)Maini R; Nguyen D; Chen S; Dedkova LM; Roy Chowdhury S; Alcalá-Torano R; Hecht SM Incorporation of β -amino acids into dihydrofolate reductase by ribosomes having modifications in the peptidyltransferase center. *Bioorg. Med. Chem.* 2013, 21, 1088–1096. [PubMed: 23375097] (c)Maini R; Roy Chowdhury S; Dedkova LM; Roy B; Daskalova SM; Paul R; Chen S; Hecht SM Protein synthesis with ribosomes selected for the incorporation of β -amino acids. *Biochemistry* 2015, 54, 3694–3706. [PubMed: 25982410]
- (3) (a). Maini R; Dedkova LM; Paul R; Madathil MM; Roy Chowdhury S; Chen S; Hecht SM Ribosome-mediated incorporation of dipeptides and dipeptide analogues into proteins *in vitro*. *J. Am. Chem. Soc.* 2015, 137, 11206–11209. [PubMed: 26301427] (b)Roy Chowdhury S; Maini R; Dedkova LM; Hecht SM Synthesis of fluorescent dipeptidomimetics and their ribosomal incorporation into green fluorescent protein. *Bioorg. Med. Chem. Lett.* 2015, 25, 4715–4718. [PubMed: 26351043]
- (4). Roy Chowdhury S; Chauhan P; Dedkova LM; Bai X; Chen S; Talukder P; Hecht SM Synthesis and evaluation of a library of fluorescent dipeptidomimetic analogues as substrates for modified bacterial ribosomes. *Biochemistry* 2016, 55, 2427–2440. [PubMed: 27050631]
- (5). Placement of the same oxazole at GFP position 39, outside of the β -barrel, gave a protein only twice as bright as wild-type GFP, suggesting the importance of protein secondary/tertiary structure on fluorescence intensity.
- (6) (a). Chalfie M; Tu Y; Euskirchen G; Ward WW; Prasher DC Green fluorescent protein as a marker for gene expression. *Science* 1994, 263, 802–805. [PubMed: 8303295] (b)Tsien RY The green fluorescent protein. *Annu. Rev. Biochem.* 1998, 67, 509–544. [PubMed: 9759496]
- (7). Piston DW; Kremers G-J Fluorescent protein FRET: the good, the bad and the ugly. *Trends Biochem. Sci.* 2007, 32, 407–414. [PubMed: 17764955]
- (8) (a). Chin JW; Cropp TA; Anderson JC; Mukherji M; Zhang Z; Schultz PG An expanded eukaryotic genetic code. *Science* 2003, 301, 964–967. [PubMed: 12920298] (b)Hendrickson TL; de Crecy-Lagard V; Schimmel P Incorporation of nonnatural amino acids into proteins. *Annu. Rev. Biochem.* 2004, 73, 147–176. [PubMed: 15189139] (c)Ngo JT; Tirrell DA Noncanonical amino acids in the interrogation of cellular protein synthesis. *Acc. Chem. Res.* 2011, 44, 677–685. [PubMed: 21815659]
- (9). Schepartz, Söll, and co-workers have used modified ribosomes² to incorporate β^3 -phenylalanine analogues into DHFR utilizing recognition by wild-type *E. coli* phenylalanyl-tRNA synthetase: Melo Czekster C; Robertson WE; Walker AS; Söll D.; Schepartz A *In vivo* biosynthesis of a β -amino acid-containing protein. *J. Am. Chem. Soc.* 2016, 138, 5194–5197. [PubMed: 27086674]
- (10) (a). Nozawa K; O'Donoghue P; Gundllapalli S; Araisio Y; Ishitani R; Umehara T; Söll D; Nureki O Pyrrolysyl-tRNA synthetase-tRNA(Pyl) structure reveals the molecular basis of orthogonality. *Nature* 2009, 457, 1163–1167. [PubMed: 19118381] (b)Kobayashi T; Yanagisawa T; Sakamoto K; Yokoyama S Recognition of non-alpha-amino substrates by pyrrolysyl-tRNA synthetase. *J. Mol. Biol.* 2009, 385, 1352–1360. [PubMed: 19100747] (c)Wan W; Tharp JM; Liu WR Pyrrolysyl-tRNA synthetase: an ordinary enzyme but an outstanding genetic code expansion tool. *Biochim. Biophys. Acta, Proteins Proteomics* 2014, 1844, 1059–1070.
- (11). Trott O; Olson AJ AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 2009, 31, 455–461.
- (12). The AutodockTools, which is the graphic interface of Autodock Vina, was used for definition of the binding site. The search was carried out within a $60 \times 60 \times 60$ Å box. Flexible torsion angles were assigned to the ligands. An I7-7700HQ CPU with eight logical cores was used for the calculation, and the exhaustiveness of each calculation was set at 30. The results were analyzed further using PyMOL version 2.1.1.
- (13). S-30s preparations having modified ribosomes with this mutation (rrnB operon with nucleotide sequences 2057UGCGUGG2063 and 2502ACGAAG2507 in the 23S rRNA gene) gave the best suppression yields during *in vitro* protein synthesis with oxazole **1**.^{3a}

- (14). Bai X; Talukder P; Daskalova SM; Roy B; Chen S; Li Z; Dedkova LM; Hecht SM Enhanced binding affinity for an i-motif DNA substrate exhibited by a protein containing nucleobase amino acids. *J. Am. Chem. Soc.* 2017, 139, 4611–4614. [PubMed: 28263595]
- (15). The quantum yield for BFP is 0.24.^{6b} Analysis of the three samples by PAGE indicated the same relative sensitivity to fluorescence detection (not shown).
- (16). Gunning PW; Ghoshdastider U; Whitaker S; Popp D; Robinson RC The evolution of compositionally and functionally distinct actin filaments. *J. Cell Sci.* 2015, 128, 2009–2019. [PubMed: 25788699]
- (17) (a). Erickson H Cytoskeleton. Evolution in bacteria. *Nature* 2001, 413, 30. [PubMed: 11544510] (b)Popp D; Narita A; Maeda K; Fujisawa T; Ghoshdastider U; Iwasa M; Maéda Y; Robinson RC. Filament structure, organization, and dynamics in MreB sheets. *J. Biol. Chem.* 2010, 285, 15858–15865. [PubMed: 20223832] (c)Swulius MT; Chen S; Ding HJ; Li Z; Briegel A; Pilhofer M; Tocheva EI; Lybarger SR; Johnson TL; Sandkvist M; Jensen GJ Long helical filaments are not seen encircling cells in electron cryotomograms of rod-shaped bacteria. *Biochem. Biophys. Res. Commun.* 2011, 407, 650–655. [PubMed: 21419100] (d)Garner EC; Bernard R; Wang W; Zhuang X; Rudner DZ; Mitchison T Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science* 2011, 333, 222–225. [PubMed: 21636745] (e)Dominguez-Escobar J; Chastanet A; Crevenna AH; Fromion V; Wedlich-Söldner; Carballido-Lopez R. Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria. *Science* 2011, 333, 225–228. [PubMed: 21636744]
- (18) (a). Kruse T; Bork-Jensen J; Gerdes K The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. *Mol. Microbiol.* 2005, 55, 78–89. [PubMed: 15612918] (b)Divakaruni AV; Baida C; White CL; Gober JW The cell shape proteins MreB and MreC control cell morphogenesis by positioning cell wall synthetic complexes. *Mol. Microbiol.* 2007, 66, 174–188. [PubMed: 17880425]
- (19). Swulius MT; Jensen GJ The helical MreB cytoskeleton in *Escherichia coli* MC1000/pLE7 is an artifact of the N-terminal yellow fluorescent protein tag. *J. Bacteriol.* 2012, 194, 6382–6386. [PubMed: 22904287]
- (20). The plasmid was obtained from a commercial supplier (Synbo Technology).
- (21) (a). Van den Ent F; Amos LA; Löwe J. Prokaryotic origin of the actin cytoskeleton. *Nature* 2001, 413, 39–44. [PubMed: 11544518] (b)Gaballah A; Kloeckner A; Otten C; Sahl H; Henrichfreise B Functional analysis of the cytoskeleton protein MreB. *PLoS One* 2011, 6, e25129. [PubMed: 22022378]
- (22) (a). Esue O; Cordero M; Wirtz D; Tseng Y The assembly of MreB, a prokaryotic homolog of actin. *J. Biol. Chem.* 2005, 280, 2628–2635. [PubMed: 15548516] (b)Van den Ent F; Jonson CM; Persons L; de Boer P; Löwe J. Bacterial actin MreB assembles in complex with cell shape protein RodZ. *EMBO J.* 2010, 29, 1081–1090. [PubMed: 20168300] (c)Laddomada F; Miyachiro MM; Dessen A Structural insights into protein-protein interactions involved in bacterial cell wall biogenesis. *Antibiotics (Basel, Switz.)* 2016, 5, 14.
- (23) (a). Shih YL; Rothfield L The bacterial cytoskeleton. *Microbiol. Mol. Biol. Rev.* 2006, 70, 729–754. [PubMed: 16959967] (b)van Teeffelen S; Wang S; Furchtgott L; Huang KC; Wingreen NS; Shaevitz JW; Gitai Z The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. *Proc. Natl. Acad. Sci. U. S. A.* 2011, 108, 15822–15827. [PubMed: 21903929]
- (24). While the photophysical properties of current oxazoles do not permit high resolution fluorescence microscopy to be carried out, the identification of modified fluorophores having more appropriate properties is a current focus of our studies.

oxazole 1

oxazole 2

p-cyanophenylalanine**Figure 1.**

Structures of noncanonical amino acids incorporated into proteins *in vitro* and *in cellulo*.

Figure 2. Characterization of RRM1s, prepared *in cellulo*. (A) SDS-PAGE analysis; Coomassie Brilliant Blue visualization. Ten microliter aliquots of CNPhe- and oxazole **2**-containing samples were analyzed. (B) Emission spectra for RRM1 containing CNPhe (blue trace, 2 μ M, excitation at 300 nm) and oxazole **2** (orange trace, 0.4 μ M, excitation at 350 nm). A control employed free oxazole **2** (gray trace, 2 μ M, excitation at 350 nm). M = MW markers of 10, 15, 20, 25, 37, and 50 kDa.

Figure 3.

(A) SDS-PAGE analysis of GFP containing oxazole **2** (position 66) and RRM1 containing **2** (position 24). (B) Fluorescence emission spectra (excitation at 350 nm). Orange trace, GFP-66-**2** (1 μ M); blue trace, RRM1-24-**2** (1 μ M); gray, free oxazole **2** (10 μ M). M = MW markers as in Figure 2.

Figure 4.

SDS PAGE analysis of MreB, obtained after *in vivo* expression in BL-21-Pyl-rrnB010328R4 cells, following purification by Strep-tactin chromatography. MreB-wt, synthesis from wild-type gene; MreB-13 **2**, synthesis from gene having TAG codon (position 13) in the presence of **2** mM oxazole **2**. M = MW markers at 10, 20, 37, and 50 kDa.

Figure 5.

Microscopic study of cells, hosting MreB-wt (prepared in the presence or absence of oxazole **2**), mutant MreB having oxazole **2** at position 13, and BFP. The images were obtained using a Nikon Eclipse Ti2 Inverted Microscope System. The excitation wavelength was 357 ± 22 nm; the emission wavelength was 435 ± 25 nm. The inset shows an enlargement of the image of cells harboring the plasmid encoding modified MreB and grown in the presence of oxazole **2**. The BFP control lacked **2**.