A New Strategy for the Site-Specific Modification of Proteins *in Vivo*†

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ABSTRACT: We recently developed a method for genetically incorporating unnatural amino acids site-specifically into proteins expressed in *Escherichia coli* in response to the amber nonsense codon. Here we describe the selection of an orthogonal tRNA−TyrRS pair that selectively and efficiently incorporates *m*-acetyl-L-phenylalanine into proteins in *E. coli*. We demonstrate that proteins containing *m*-acetyl-L-phenylalanine or *p*-acetyl-L-phenylalanine can be selectively labeled with hydrazide derivatives not only *in vitro* but also in living cells. The labeling reactions are selective and in general proceed with yields of >75%. In specific examples, *m*-acetyl-L-phenylalanine was substituted for Lys7 of the cytoplasmic protein Z domain, and for Arg200 of the outer membrane protein LamB, and the mutant proteins were selectively labeled with a series of fluorescent dyes. The genetic incorporation of a nonproteinogenic "ketone handle" into proteins provides a powerful tool for the introduction of biophysical probes for the structural and functional analysis of proteins *in vitro* or *in vivo*.

The site-specific modification of proteins with biophysical probes (*1*, *2*), cytotoxic agents (*3*−*6*), cross-linking agents (*7*, *8*), and other agents (*9*) has been widely used to analyze protein structure (*10*) and function (*11*), and in the development of diagnostics (*3*), therapeutic agents (*12*), and high-throughput screens (*13*). One approach to the selective modification of proteins involves the derivatization of cysteines with thiol-specific maleimide-based or haloacetamide-based reagents (*14*, *15*). Selective modification, however, requires a single reactive cysteine which is often not the case with larger proteins. A second strategy involves the oxidation of an N-terminal serine or threonine to the corresponding aldehyde and subsequent coupling with hydrazine, alkoxyamine, or hydrazide derivatives (*16*). Unfortunately, this method is limited since it can only be used to modify the N-terminal position of a protein, the protein must be stable to oxidation, and the yields can be variable. A third strategy, semisynthesis (*17*), has been used to site-specifically incorporate novel amino acids into proteins. In this method, segments of proteins are synthesized by solid phase peptide synthesis (SPPS) and then chemically (*18*, *19*) or enzymatically (*20*−*24*) ligated. Unfortunately, it is difficult to synthesize proteins of more than 100−200 residues using this method (*17*, *25*). Expressed protein ligation (EPL) has been developed to access larger polypeptides with some success (*26*, *27*). EPL involves the ligation of a synthetic peptide with an N-terminal cysteine to a recombinant protein. Unfortunately, incorporation of the novel amino acid is limited to the C-terminus of the protein.

To avoid the difficulties associated with chemical methods, an *in vitro* biosynthetic method has been developed to incorporate unnatural amino acids site-specifically into proteins (*28*, *29*). An amber suppressor tRNA[1] which is not a substrate for the endogenous aminoacyl-tRNA synthetases is chemically modified with the desired amino acid. Addition of this acylated suppressor tRNA and a gene containing an amber stop codon at the site of interest to an *in vitro* transcription/translation system yields the desired mutant protein containing the unnatural amino acid. Using this method, Cornish et al. incorporated a keto-containing amino acid selectively into proteins *in vitro* (*30*), which was subsequently labeled with hydrazide-containing fluorescent probes. In aqueous solution, the keto group reacts with hydrazide and alkoxyamine derivatives to form hydrazones and oximes, respectively (*16*, *31*). The products are stable under physiological conditions and are formed selectively in the presence of other functional groups present in proteins. Unfortunately, *in vitro* protein synthesis typically produces only small amounts of protein. Moreover, the preparation of the acylated tRNA, which is consumed stoichiometrically during translation and cannot be regenerated, can only be carried out on relatively small scales.

Recently, an *in vivo* method was developed to incorporate unnatural amino acids site-specifically into proteins in *Escherichia coli* with high fidelity and efficiency (*32*). It involves the generation of a new tRNA−synthetase pair

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[1] Abbreviations: tRNA, transfer ribonucleic acid; TyrRS, tyrosyl-tRNA synthetase; TFA, trifluoroacetic acid; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; t_R, retention time.

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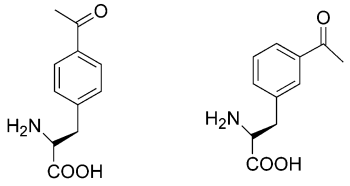
which can site-specifically insert the unnatural amino acid of interest into proteins in *E. coli* in response to the amber codon. This tRNA−synthetase pair is orthogonal to its counterparts for the common 20 amino acids; i.e., this synthetase, and only this synthetase aminoacylates the orthogonal tRNA, and only this tRNA with the unnatural amino acid and no other amino acids. The resulting acylated tRNA then incorporates the unnatural amino acid only in response to the amber codon, TAG. Using this approach mutant proteins can be conveniently produced in large quantity. Unlike semisynthetic methods, there is no size limitation on the mutant proteins, and the unnatural amino acid can be substituted at any position. In addition, since the mutant protein is synthesized endogenously, it may be possible to carry out the selective modification of proteins in living cells. To date, a number of unnatural amino acids have been successfully incorporated into proteins, including *p*- and *m*-methoxy-L-phenylalanine (*32*), L-2-naphthylalanine (*33*), *O*-allyl-L-tyrosine (*34*), *p*-azido-L-phenylalanine (*35*), *p*-benzoyl-L-phenylalanine (*36*), *p*-amino-L-phenylalanine (*37*), *p*-isopropyl-L-phenylalanine (*37*), *p*-iodo-L-phenylalanine, and *p*-bromo-L-phenylalanine.

Recently, we showed that it was possible to use this methodology to incorporate the keto amino acid, *p*-acetyl-L-phenylalanine **1**, into proteins in response to the amber nonsense codon (*38*). This amino acid could subsequently be selectively modified with hydrazide (*38*) and alkoxyamine derivatives in high yield (*39*). Herein, we extend this methodology to *m*-acetyl-L-phenylalanine **2** and show that proteins containing *m*-acetyl-L-phenylalanine can be selectively labeled with hydrazide derivatives not only *in vitro* but also *in vivo* (*40*−*42*). Asp200 of the outer membrane protein LamB, and Lys7 of a cytoplasmically expressed Z domain protein were specifically mutated to *m*-acetyl-L-phenylalanine **2**. The incorporation of the unnatural *m*-acetyl-L-phenylalanine did not impair either protein's biological functions, and the resulting mutant proteins containing a "ketone handle" were selectively labeled *in vivo* with hydrazide-derivatized fluorescent dyes.



1 **2**

MATERIALS AND METHODS

Materials. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Aldrich. IPTG (β-D-isopropylthiogalactose) was purchased from Sigma. Dodecyl maltoside was purchased from Anatrace Inc. (Maumee, OH). Fluorescein hydrazide, biotin hydrazide, Cascade blue hydrazide trisodium salt, Alexa568 hydrazide sodium salt, and Alexa647 hydrazide sodium salt were purchased from Molecular Probes (Eugene, OR). All aqueous solutions were prepared from doubly distilled water filtered with a Milli-Q purification system. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). PCRs were carried out using the High Fidelity Expansion Kit from Roche (Indianapolis, IN). Site-directed mutagenesis was carried out using the Quikchange kit from Stratagene (La Jolla, CA). Ni^{2+}−NTA beads were purchased from Qiagen (Valencia, CA). PD-10 columns were purchased from Amersham Pharmacia (Piscataway, NJ). Fluorescence emission spectra were recorded on a FluoroMax-2 spectrometer (Packard Instrument Co.). DNA purification was carried out using Qiagen kits. Microscopic imaging experiments were carried out on a DeltaVision Deconvolution Restoration Microscope system running SoftWoRx version 2.5 (Applied Precision Inc., Issaquah, WA). ^1H and ^{13}C NMR spectra were recorded using a Bruker AMX-400 spectrometer. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilane using the residual solvent as the internal standard. Coupling constants are expressed in hertz.

Synthesis of m-Acetyl-(±)-phenylalanine. NBS (*N*-bromosuccinimide) was recrystallized from water prior to use. NBS (18.5 g, 105 mmol) was added to a solution of 3-methylacetophenone (13.4 g, 100 mmol) in 400 mL of carbon tetrachloride, followed by addition of AIBN (2′,2′-azobisisobutyronitrile) (0.43 g, 2.5 mmol). The reaction mixture was then heated to reflux for 4 h. After completion of the reaction (TLC, 8:1 hexanes/EtOAc mixture), the solution was washed with water (1 × 100 mL), 1 M aqueous HCl (3 × 100 mL), 0.5% aqueous $NaHCO_3$ (3 × 100 mL), and brine (1 × 100 mL). The organic layer was collected and dried over anhydrous $MgSO_4$. After removal of the $MgSO_4$ by filtration, the organic solvent was evaporated to obtain a yellow solid. This solid was recrystallized with hexanes to afford the desired 1-[(3-bromoethyl)phenyl]ethanone as a brown solid (18.3 g, 85% yield).

Dry ethanol (500 mL) was added dropwise with stirring to pentane-washed pieces of sodium (23 g, 1 mol) under an argon atmosphere over the course of 15 min. The solution was stirred for an additional 15 min to dissolve the last pieces of sodium. Solid diethyl acetamidomalonate (27 g, 100 mmol) was then added over the course of 45 min with stirring followed by the dropwise addition of 1-[(3-bromoethyl)-phenyl]ethanone (21 g, 100 mmol) in dry ethanol over the course of 90 min. After the mixture was heated to reflux overnight, diethyl ether (500 mL) and water (500 mL) were slowly added to the solution. The organic layer was separated and washed successively with 0.5% aqueous $NaHCO_3$ (3 × 300 mL) and brine (1 × 300 mL). The solution was dried over anhydrous $MgSO_4$ and filtered, and the solvent was removed *in vacuo* to afford a brown gummy solid. A mixture of hexanes and dichloromethane (4:1) was added to the residue, and the insoluble material was recovered and washed exhaustively with a 10:1 dichloromethane/benzene mixture to afford 2-(acetylamino)-2-(3-acetylbenzyl)malonic acid diethyl ester as a yellow solid (33 g, 95% crude yield). This compound was stirred with 4 M anhydrous HCl in dioxane overnight. The reaction was quenched with water, the mixture was then evaporated to dryness and recrystallized from water to afford *m*-acetylphenylalanine (16.0 g, 77% overall yield) as a white solid: mp 198.2−200.0 °C; ^1H NMR (D_2O) δ 7.85−7.04 (m, 4H), 4.27 (dd, 1H, J = 5.4), 3.30 (m, 2H), 2.55 (s, 3H); ^{13}C NMR (D_2O) δ 198.5, 176.6, 136.3, 131.8,

129.9, 128.4, 126.6, 57.8, 39.5, 26.5; LRMS calcd for $C_{11}H_{13}NO_3$ ($M^+ + 1$) 208.09, found 208.07.

Synthesis of p-Acetyl-(±)-phenylalanine. The procedures for synthesizing *p*-acetyl-(±)-phenylalanine (*38*) from 4-methylacetophenone were identical to those for synthesizing *m*-acetyl-(±)-phenylalanine. *p*-Acetyl-(±)-phenylalanine was obtained as a white solid (13.2 g, 64% overall yield): mp 206.8−207.5 °C; ^{1}H NMR (400 MHz, D_2O) δ 7.85−7.28 (m, 4H), 4.27 (dd, 1H, $J = 5.4$), 3.30 (m, 2H), 2.68 (s, 3H); ^{13}C NMR (D_2O) δ 195.8, 174.3, 145.9, 133.1, 128.9, 127.8, 60.2, 38.3, 26.5; LRMS calcd for $C_{11}H_{13}NO_3$ ($M^+ + 1$) 208.09, found (ESI) 208.07.

Plasmids and Cell Lines. Strain GS20 (*flhd fruA relA1 rpsL rpoB thi metA lamB20 deoC1*) was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). Plasmid pLEIZ, encoding both mu $tRNA^{Tyr}_{CUA}$ under the control of the *lpp* promoter and *rrnC* terminator and the Z domain protein gene with an amber codon at the seventh position under the control of a bacteriophage *T5* promoter and t_0 terminator was prepared previously (*34*). Plasmid pBK-JYRS encoding the wild-type *Methanococcus jannaschii* tyrosyl-tRNA synthetase (TyrRS) under the control of the *E. coli* GlnRS promoter and terminator was prepared previously and used here (*32*). Plasmid pREP(2)/YC, encoding mu $tRNA^{Tyr}_{CUA}$, chloramphenicol acetyltransferase (CAT) gene with an amber TAG at Asp112 and GFP gene under the control of the T7 promoter and its cognate amber mutant T7 RNA polymerase, was constructed previously. *E. coli* DH10B harboring pREP(2)/YC was used as the host strain for the positive selection (*37*). Plasmid pLWJ17B3, expressing mu $tRNA^{Tyr}_{CUA}$ under the control of the *lpp* promoter and *rrnC* terminator, and the barnase gene with three amber codons at Gln2, Asp44, and Gly65 under the control of the *ara* promoter, was constructed previously (*32*). *E. coli* DH10B harboring pLWJ17B3 was used as the host strain for negative selection.

The LamB gene was amplified from *E. coli* genomic DNA by PCR using the following primers: LamB-top, 5′-GCCTGGAGATGATGATTACTCTGCGCAA-3′; and LamB-bot, 5′-CGGGTACCCCACCAGATTTCCATCTGGGC-3′. The fragment was inserted into vector pBAD/JYCUA which encodes mu $tRNA^{Tyr}_{CUA}$. The resulting plasmid pLamB encodes both the wild-type LamB protein and mu $tRNA^{Tyr}_{CUA}$ under the control of the *ara* promoter. The sequences of oligonucleotides for site-directed mutagenesis were as follows: LamB-mu-top, 5′-ACGACGTTTTCTAGGTGCGT-3′; and LamB-mu-bot, 5′-ACGCACCTAGAAAACGTCGT-3′. This process generated the plasmid pLamB200mAcPhe with the codon for Asp200 of LamB mutated to TAG.

Construction of the MjTyrRS Libraries. Plasmid DNA (pBK-lib) (*32*) encoding a library of *M. jannaschii* TyrRS variants randomized at residues Tyr32, Glu107, Asp158, Ile159, and Leu162 was previously constructed, resulting in approximately 1.6×10^9 independent clones. This library was used to select for mutant TyrRS enzymes that can specifically charge para-substituted tyrosine analogues. In a similar fashion, plasmid DNA (pBK-lib-m) encoding a library of *M. jannachii* TyrRS mutants for selection with meta-substituted phenylalanine was created by an overlapping fragment PCR approach using the following primers: JZNdeI (5′-terminal primer), 5′-CGCATATGATGGACGAATTTGAAATGAT-3′; JZY32/ALA or NNK, 5′-TACCACTTGGTTCAAAACCTATCGCAGCAGATTTTTCA-3′ or 5′-TACCACTTGGTTCAAAACCTATMNNAGCAGATTTTTCA-3′, respectively; JZ03, 5′-GTTTTGAACCAAGTGGTA-3′; JZA67H70/ALA or NNK, 5′-CCTTTCTGGTTTAAATAGGCCGCTAAATCAGCCAACAATA-3′ or 5′-CCTTTCTGGTTTAAATAGGCMNNTAAATCMNNCAACAATA-3′, respectively; JZ04, 5′-GCCTATTTAAACCAGAAAGG-3′; JZQ155D158/ALA or NNK, 5′-CAACATCAACGCCTAAATAATGAATCGCATTAACCGCCATTATTGGA-3′ or 5′-CAACATCAACGCCTAAATAATGAATMNNATTAACMNNCATTATTGGA-3′, respectively; JZA167/ALA or NNK, 5′-TATTTAGGCGTTGATGTTGCAGTTGGAGGG-3′ or 5′-TATTTAGGCGTTGATGTTNNKGTTGGAGGG-3′, respectively; JZPstI (3′-terminal primer), 5′-AAACTGCAGTTATAATCTCTTTCTAATTGGCTC-3′.

Turbo pfu polymerase (Stratagene) was used for all PCRs according to the manufacturer's protocol. First, four fragments were amplified by PCR from pBK-JYRS to introduce alanine mutations at Tyr32, Ala67, His70, Gln155, Asp158, and Ala167 in *M. jannachii* TyrRS. The two 5′-terminal fragments were then diluted, combined, and subjected to PCR with the end-most primers (JZNdeI and JZA67/H70/ALA), and likewise for the C-terminal fragments. These two larger fragments were then diluted, combined, and amplified with the JZNdeI and JZPstI primers. The final fragment was then digested with restriction enzymes *Nde*I and *Pst*I, purified, and ligated back into the pBK vector to afford plasmid pBK-JYRS-ALA. Using pBK-JYRS-ALA as the template and doped NNK and NNM primers, the same procedures described above were repeated to introduce the randomized triplet codon (NNK) at Ala32, Ala67, Ala70, Ala155, Ala158, and Ala167. The resulting plasmids (pBK-lib-m) encode the library of *M. jannaschii* TyrRS variants, randomized at positions Tyr32, Ala67, His70, Gln155, Asp158, and Ala167. This library contains approximately 1×10^9 independent transformants.

Genetic Selection of Mutant TyrRS Enzymes. The positive selection was carried out by transforming plasmid pBK-lib-m which encodes the library of TyrRS mutants described above, into competent *E. coli* DH10B cells harboring the pRep(2)/YC plasmid. The transformed cells were combined and grown in 500 mL of LB medium supplemented with 40 μg/mL tetracycline and 50 μg/mL kanamycin at 37 °C until the OD_{595} was approximately 1.0. Cells from 10 mL of this culture were harvested, washed three times with GMML medium (1× M9 minimal medium containing 1% glycerol, 0.3 mM leucine, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.5% NaCl), and resuspended in 500 mL of GMML medium containing 40 μg/mL tetracycline, 50 μg/mL kanamycin, 68 μg/mL chloramphenicol, and 1 mM *m*-acetylphenylalanine. The culture medium was incubated at 37 °C with shaking for 60 h. Surviving cells were harvested, and plasmid DNA was isolated and purified with a Qiagen maxi-prep kit. Plasmids (pBK) encoding TyrRS mutants (~2.5 kb) were separated from pRep(2)/YC using gel electrophoresis on a 1% agarose gel and gel-extracted using the Qiagen gel extraction kit. To begin the first negative selection, the resulting plasmid DNA from the positive selection was transformed into *E. coli* DH10B harboring pLWJ17B3. The transformed cells were grown in 10 mL of LB medium containing 34 μg/mL chloramphenicol and 50 μg/mL kana-

mycin at 37 °C until the OD_{595} was 0.8. Cells were then plated onto LB (Luria-Bertani) plates (10^7 cells/60 cm plate) containing 40 μg/mL chloramphenicol, 50 μg/mL kanamycin, and 0.2% arabinose. The plates were incubated at 37 °C for 8 h. Surviving cells were collected and amplified in 500 mL of LB medium with 40 μg/mL chloramphenicol and 50 μg/mL kanamycin at 37 °C for 2 h. Plasmid DNA was then isolated and purified with a Qiagen maxi-prep kit. Plasmids (pBK) encoding TyrRS mutants (~2.5 kb) were separated from pLWJ17B3 using gel electrophoresis and gel extraction with the Qiagen gel extraction kit. The second positive selection was carried out on plates. The isolated pBK plasmids from the first negative selection were transformed back into the positive selection strain. The transformed cells were collected and grown in LB medium containing 40 μg/mL tetracycline and 50 μg/mL kanamycin at 37 °C until the OD_{595} was 1.0. Cells were collected, washed with GMML three times, and plated onto GMML agar plates (10^7 cells/60 cm plate) containing 40 μg/mL tetracycline, 50 μg/mL kanamycin, 68 μg/mL chloramphenicol, and 1 mM *m*-acetylphenylalanine. Plates were incubated at 37 °C for 60 h. Surviving cells were pooled and amplified in 500 mL of LB medium with 40 μg/mL tetracycline and 50 μg/mL kanamycin. Plasmid DNA was isolated and purified with a Qiagen maxi-prep kit. The pBK plasmid DNA encoding the TyrRS mutants was separated from pRep(2)/YC by gel electrophoresis on a 1% agarose gel and used for further rounds of selection. In brief, the second negative selection and the third positive selection were performed on plates as described above. Following the third positive selection, surviving cells were examined under long-wavelength UV to confirm the presence of GFP. Colonies (48 or 96) were inoculated into 0.5 mL of LB medium containing 40 μg/mL tetracycline and 50 μg/mL kanamycin. Cultures were grown at 37 °C overnight with vigorous shaking. The saturated cultures (1 μL) were diluted into 5 mL of GMML medium. The resulting cell suspensions (1.5 μL) were plated on GMML plates containing 1 mM *m*-acetylphenylalanine, 40 μg/mL tetracycline, 50 μg/mL kanamycin, and chloramphenicol (concentrations ranging from 80 to 140 μg/mL), and GMML plates were supplemented with 40 μg/mL tetracycline, 50 μg/mL kanamycin, and chloramphenicol (concentrations ranging from 10 to 40 μg/mL). The plates were incubated at 37 °C for 60 h. Surviving cells were imaged under UV for GFP expression.

After the above selection, five orthogonal tRNA−synthetase candidate pairs were isolated. Growth of cells transformed with these orthogonal pairs was inhibited by 20 μg/mL chloramphenicol (IC_{50} = 20 μg/mL Cm) in the absence of the unnatural amino acid and 100 μg/mL chloramphenicol in the presence of 1 mM *m*-acetylphenylalanine. The plasmids encoding the five synthetases were then purified and used as templates to make two secondary TyrRS libraries (*vide infra*). The two libraries were then combined and subjected to the same selection process. Two candidate orthogonal pairs were isolated. Cells containing either one of the two candidate pairs afforded an IC_{50} of 10 μg/mL chloramphenicol in the absence of the unnatural amino acid and 120 μg/mL chloramphenicol in the presence of 1 mM *m*-acetylphenylalanine. The plasmids encoding mutant synthetases were purified, and DNA sequencing revealed that these two plasmids encoded the same mutant TyrRS (termed pmAcPheRS). Plasmid pBK-LW1RS encoding the mutant TyrRS that specifically charges *p*-acetyl-L-phenylalanine was selected from plasmids pBK-lib as described previously (*38*).

Preparation of Secondary TyrRS Libraries by DNA Shuffling and Error-Prone PCR. The five candidate TyrRS genes isolated from genetic selection were combined and were subjected to DNA shuffling as described previously (*32*, *43*). In brief, fragments containing the five mutant TyrRS genes were obtained by digesting the five isolated pBK plasmids with the enzymes *Pst*I and *Nde*I followed by gel purification. The desired 1 kb products (1 μg each) were pooled and digested with DNaseI to fragments approximately 100 bp in length. These fragments were reassembled into a full-length gene with primer LW157 (5′-GGAATTCCATATGGACGAATTTGAAATG-3′) and primer LW105 (5′-AAACTGCAGTTATAATCTCTTTCTAATTGGCTC-3′). The full-length genes were purified by electrophoresis and digested with the enzymes *Nde*I and *Pst*I. After gel purification, the desired fragments were inserted back into the *Nde*I and *Pst*I precut pBK-JYA5 vector (*32*). The resulting plasmid DNA (pBK-shuffle) contained a library of approximately 1.5×10^8 independent transformants.

Another library of random TyrRS mutants was constructed by error-prone PCR (*45*) using 0.01, 0.15, 0.25, or 0.5 mM Mn^{2+}. In this case, the five plasmids encoding candidate TyrRS mutants were used as templates for error-prone PCRs separately. Oligonucleotides JZNdeI and JZPstI were used as PCR primers. The PCR products were combined and digested with the enzymes *Nde*I and *Pst*I. After gel purification, the resulting double-strand oligonucleotides (~2.5 kb) were ligated into vector pBK. The resulting plasmid DNA (pBK-error) encoded a library of randomized TyrRS mutants containing approximately 4×10^7 independent transformants. Finally, plasmids pBK-shuffle and pBK-error were combined to form a secondary library of randomized TyrRS mutants that was used for further genetic selection as described above.

Expression of the Mutant Z Domain Protein. Plasmid pmAcPheRS was cotransformed with plasmid pLEIZ into *E. coli* TOP10 cells. A single colony was inoculated into 50 mL of LB with kanamycin (50 μg/mL) and chloramphenicol (48 μg/mL) and grown to saturation. The cells were harvested by centrifugation and washed twice with GMML medium. The pellet was resuspended in 50 mL of GMML medium, and 25 mL of this suspension was inoculated into 1 L of GMML medium. *m*-Acetylphenylalanine was added to a final concentration of 1 mM, and cells were grown at 37 °C for ~30 h to an OD_{595} of 0.5. IPTG (β-D-isopropylthiogalactose) was added to a final concentration of 1 mM, and cells were grown at 37 °C for an additional 8 h. The cells were pelleted, and the protein was purified by affinity chromatography with Ni^{2+}−NTA beads under denaturing conditions according to the manufacturer's protocol (Qiagen). Proteins were then desalted with a PD-10 column (Amersham Pharmacia) and eluted in water. Purified protein (5 μg) from the positive and negative controls was separated on a denaturing 12% Tris-HCl−polyacrylamide gel and stained with Coomassie blue. The yield of the Lys7 → *m*-acetylphenylalanine mutant Z domain protein was 1.8 mg/L of culture. An aliquot of purified protein was subjected to high-resolution mass spectrometric analysis.

In Vitro Labeling of the Mutant Protein with Fluorescein Hydrazide. The following procedure was used to label mutant proteins containing either *m*-acetyl-L-phenylalanine or *p*-acetyl-L-phenylalanine. The purified Z domain protein (1 mg/mL) was exchanged into 1× PBS [100 mM potassium phosphate (pH 7.0) and 0.5 M sodium chloride] by dialysis. The labeling reactions were carried out in 2 mL silanized Eppendoff tubes. Fluorescein hydrazide (0.45 mg, 1 mM) was dissolved in 200 μL of DMF followed by addition of 600 μL of 1× PBS. Z domain protein (50 μg, 2 μM) was then added to the solution, and 1× PBS buffer was added to make the final volume 1 mL. The reaction mixture was gently tumbled at 4 °C for 12 h. The labeled protein was dialyzed in 1× PBS for 24 h at 4 °C, desalted using a PD-10 column (Amersham Pharmacia), and eluted in 1× PBS buffer. The protein concentration was estimated to be 0.2 μg/μL by SDS−PAGE, silver staining, and comparison to protein standards. To examine the labeling efficiency, the desalted protein mixture was further analyzed by reverse phase HPLC (Agilent ZORBAX SB-C18 column, 4.6 mm × 250 mm, flow rate of 1.0 mL/min, from 10 to 40% CH_3CN in aqueous 50 mM triethylammonium acetate at pH 7.0 over the course of 70 min).

Fluorescence Measurements. All fluorescence emission spectra were recorded using a FluoroMax-2 fluorimeter with excitation at 490 nm, excitation and emission band-passes of 4 and 8 nm, respectively, a PMT voltage of 950 V, and a scan rate of 1 nm/s. The reported spectra are uncorrected.

In Vivo Labeling of the Mutant Protein with Fluorescein Hydrazide. E. coli cells (100 mL) expressing the mutant protein were pelleted and washed with 1× PBS (pH 7.0) containing 10% glycerol to remove excess unnatural amino acid. The cells were resuspended in 5 mL of 1× PBS containing 10% glycerol. Fluorescein hydrazide (4.5 mg, 1 mM) was dissolved in 500 μL of DMF and added dropwise to the cell culture suspension. Additional 1× PBS buffer (pH 7.0) containing 10% glycerol was added to bring the final volume to 10 mL. The reaction mixture was gently tumbled at 4 °C for 18 h. Cells were harvested and washed thoroughly with 1× PBS buffer (pH 7.0). Labeled Z domain-His6 protein was purified with a Ni^{2+}−NTA column under denaturing conditions according to the manufacturer's protocol (Qiagen). The protein solution was further desalted to remove excess fluorescein hydrazide with a PD-10 column (Amersham Pharmacia). The protein concentration was estimated using SDS−PAGE, silver staining, and comparison to protein standard. The eluted protein samples were further analyzed by reverse phase HPLC (Agilent ZORBAX SB-C18 column, 4.6 mm × 250 mm, flow rate of 1.0 mL/min, from 10 to 40% CH_3CN in aqueous 50 mM triethylammonium acetate buffer at pH 7.0 over the course of 70 min). The retention time (t_R) for the intact Z domain protein was 37.4 min; the t_R for the fluorescein hydrazide-labeled Z domain protein was 38.8 min. An aliquot of purified protein was subjected to high-resolution mass spectrometric analysis.

In Vivo Labeling of the Mutant Protein with Biotin Hydrazide. The procedures for *in vivo* labeling of the mutant protein with biotin hydrazide are identical to those for fluorescein hydrazide as described above. Labeled mutant Z domain His6 protein was purified using Ni^{2+}−NTA beads under denaturing conditions. The resulting proteins were separated by SDS−PAGE and then transferred to a nitrocellulose membrane. The biotin-labeled protein was stained with avidin fused to horseradish peroxidase (HRP, Bio-Rad, Hercules, CA). Substrate (SuperSignal West Dura, Pierce, Rockford, IL) was applied to visualize the signals. The proteins purified with Ni^{2+}−NTA beads were further separated by reverse phase HPLC as described above. The retention time for biotin hydrazide-labeled Z domain protein was 38.6 min.

Expression of LamB200mAcPhe Protein. GS20 cells were cotransformed with plasmids pmAcPheRS and pLamB200mAcPhe. A single colony was inoculated into 5 mL of LB medium containing 50 μg/mL kanamycin and 40 μg/mL tetracycline and grown to saturation. The cells were pelleted and washed twice with GMML medium. After the pellet was suspended in 5 mL of GMML medium, the culture was inoculated into 500 mL of GMML medium with *m*-acetylphenylalanine (1 mM) and grown at 37 °C to an OD_{595} of 0.5. Protein expression was induced by the addition of L-arabinose (0.2%), and cells were grown for an additional 4 h at 37 °C. Cells were harvested and resuspended in 10 mL of 50 mM Tris (pH 7.5) and 200 mM KCl containing protease inhibitors. The cells were lysed with a French press, and dodecyl maltoside was added to the solution to a final concentration of 40 mM. The suspension was shaken at room temperature for 1 h and centrifuged at 18000*g* for 45 min, and the supernatant was collected. Total protein (100 μg) was loaded per lane. Samples were separated on 12% SDS−PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature in Tris-buffered saline (TBS) (pH 7.5) containing 2% dry milk and then stained with anti-LamB rabbit polyclonal sera (1:2500) at 4 °C overnight. Following a series of washes with Tris-buffered saline (pH 7.5), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000 dilution, Bio-Rad) for 1 h at room temperature and washed again. Substrate (SuperSignal West Dura, Pierce) was applied to visualize the signals.

In Vivo Maltose Transport Assay. GS20 cells cotransformed with pLamB200mAcPhe and pmAcPheRS were grown in LB medium supplemented with kanamycin (50 μg/mL) and tetracycline (40 μg/mL) at 37 °C until the OD_{595} was 0.5. The cells from 2 mL of culture were harvested at 10000*g* for 5 min in an Eppendorf centrifuge. The cells were washed three times with M9 minimal medium and resuspended in the same medium. The cell suspension was adjusted to an OD_{595} of 0.5, and cells from the suspension were streaked onto plates containing 0.3 mM leucine, 1% maltose, 50 μg/mL kanamycin, 40 μg/mL tetracycline, and 1 mM *m*-acetylphenylalanine as described in Table 1. The plates were incubated at 37 °C for 60 h to evaluate cell growth rates.

In Vivo λ Phage Receptor Activity Assay. GS20 cells cotransformed with pLamB200mAcPhe and pmAcPheRS were grown to saturation in GMML medium (1% glycerol, 0.3 mM leucine, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.5% NaCl) containing 1 mM *m*-acetylphenylalanine, 50 μg/mL kanamycin, and 40 μg/mL tetracycline. Mutant λ phage K54, which is incapable of lysogeny, was used to infect these cells. Phage titer was quantitated by placing the test culture, which was incubated with 100-fold serially diluted portions of the phage K54, in an overlay on M9 minimal agar plates containing 1% glycerol, 0.3 mM leucine, 1 mM $MgCl_2$, 0.1

mM $CaCl_2$, 0.5% NaCl, 1 mM *m*-acetylphenylalanine, 50 μg/mL kanamycin, and 40 μg/mL tetracycline. The plates were incubated at 37 °C for 8 h. The plaque-forming units (pfu) were calculated by counting the number of plaques.

In Vivo Labeling for Microscopic Imaging. Cells from 2 mL of culture were pelleted and washed with 1× PBS containing 10% glycerol to remove excess *m*-acetylphenylalanine. Cells were then incubated with 1 mL of hydrazide-derivatized dyes (final concentration of 1 mM) in 1× PBS (pH 7.0) containing 10% glycerol at 4 °C overnight. Cells were washed extensively with 1× wash buffer to remove excess dye. For fluorescein hydrazide, the wash buffer consisted of 1× PBS (pH 7.0), 10% glycerol, and 10% DMF. For Cascade blue hydrazide, Alexa568 hydrazide, and Alexa 647 hydrazide, the wash buffer consisted of 1× PBS (pH 7.0) containing 10% glycerol. After washes, cells were resuspended in 1× PBS buffer (pH 7.0).

Labeled bacteria were suspended in 1× PBS, and 2 μL of the suspension was spread on a 1.5% agarose slab. Images were collected on a DeltaVision Deconvolution Restoration Microscope system running SoftWoRx version 2.5 (Applied Precision Inc.). The system was mounted on an Olympus IX70 inverted microscope with a 100× objective with an auxiliary magnification of 1.5×. Single color images were merged for the final three color images.

RESULTS AND DISCUSSION

We have investigated the genetic incorporation of two keto amino acids, *p*-acetyl-L-phenylalanine **1** and *m*-acetyl-L-phenylalanine **2**, into proteins *in vivo*. Previously, we showed that *p*-acetyl-L-phenylalanine can be site-specifically incorporated into proteins in *E. coli* in response to the TAG amber codon (*38*). Both the efficiency and fidelity of incorporation were high. *In vitro* experiments demonstrated that the incorporated keto group could be specifically labeled with biotin hydrazide and fluorescein hydrazide. To further extend this methodology, we have developed an orthogonal tRNA−synthetase pair capable of incorporating *m*-acetylphenylalanine **2** site-specifically into proteins *in vivo*. This second amino acid provides variability in the geometry of the linkage of the hydrazide and oxime analogues to proteins. Moreover, we show that this amino acid can be used to efficiently and selectively label proteins *in vitro*, and can also be used to label both membrane and cytoplasmic proteins in *E. coli*.

Evolution of TyrRS Mutants Specific for m-Acetyl-L-phenylalanine. Previously, it has been shown that the *M. jannaschii* tyrosyl-tRNA synthetase (TyrRS) and a mutant tyrosyl amber suppressor tRNA (mu $tRNA^{Tyr}_{CUA}$) are an orthogonal pair in *E. coli* (*46*). The first step in generating a new mutant TyrRS specific for *m*-acetyl-L-phenylalanine involved the design and construction of a new library of TyrRS active site mutants. This mutant TyrRS library was generated to specifically accommodate substituents at the *meta* position of phenylalanine. While the X-ray structure of *M. jannaschii* tyrosyl-tRNA synthetase (TyrRS) is not available, the crystal structure of the homologous *Bacillus stearothermophilus* TyrRS in complex with tyrosine has been determined (*47*). Using this crystal structure as a model, six residues (Tyr32, Ala67, His70, Gln155, Asp158, and Ala167) were chosen for randomization. The crystal structure suggests that residues Tyr32 and Asp158 likely form hydrogen bonds with the hydroxyl group of tyrosine (*47*). Therefore, mutations at these two sites will interrupt the hydrogen-bonded network that binds the tyrosine substrate. The other four amino acids (Ala67, His70, Gln155, and Ala67) were chosen because they are in the proximity of the *meta* position of the phenyl ring. We anticipated that mutations at these positions would be most critical for binding of tyrosine or phenyalanine derivatives with substituents at the meta position. To reduce the amount of wild-type TyrRS in the library, these six residues were mutated first to alanine and then randomized to all 20 amino acids at the DNA level using the NNK codon scheme (see Materials and Methods). The library encoded in plasmid pBK-lib-m contained around 1×10^9 independent transformants.

The next step involved the use of a genetic selection to alter the specificity of the TyrRS to specifically incorporate *m*-acetyl-L-phenylalanine (*33*−*36*). The genetic selection consists of several rounds of positive and negative selection. In the positive selection, the codon for Asp112 in the chloramphenicol acetyltransferase (CAT) gene encoded on plasmid pREP(2)/YC is replaced with an amber codon. Cells that survive high concentrations of chloramphenicol in the presence of *m*-acetylphenylalanine must express a mutant TyrRS with the ability to insert either a natural amino acid or *m*-acetyl-L-phenylalanine in response to the Asp112TAG amber codon. The negative selection is based on the toxicity of barnase, a member of the family of homologous microbial ribonucleases (*48*, *49*). All mutant TyrRS enzymes isolated from the positive selection were subjected to a negative selection in which three amber codons were placed at permissive sites of the barnase gene encoded on plasmid pLWJ17B3. In the negative selection, cells expressing mutant TyrRS mutants were selected for their ability to survive in the absence of *m*-acetylphenylalanine. Those cells expressing mutant TyrRS enzymes capable of charging natural amino acids onto mu $tRNA^{Tyr}_{CUA}$ produce full-length barnase and die. Thus, those cells expressing TyrRS mutants capable of charging *m*-acetylphenylalanine onto orthogonal mu $tRNA^{Tyr}_{CUA}$, but not natural amino acids, survive both positive and negative selections. After three rounds of positive selection and two rounds of negative selection, five candidate clones emerged. These candidate synthetase enzymes were further characterized for their efficiency and specificity in charging *m*-acetylphenylalanine *in vivo* based on the suppression of the Asp112(TAG) codon in the CAT gene (*50*). In the absence of *m*-acetylphenylalanine, cells expressing mu $tRNA^{Tyr}_{CUA}$ and one of the five mutant synthetases survived in the presence of 20 μg/mL chloramphenicol on GMML plates; in the presence of *m*-acetylphenylalanine, the same cells survived in the presence of 100 μg/mL chloramphenicol. The difference between these two numbers indicates that the activities of the selected synthetases are higher for *m*-acetylphenylalanine than for natural amino acids. However, when these five mutant TyrRS enzymes were used to express the mutant Z domain protein *in vivo*, the yield was low (data not shown).

To increase the efficiency of unnatural amino acid incorporation, two secondary libraries of mutant TyrRS enzymes derived from the isolated five candidate mutant TyrRSs were prepared with error-prone PCR (*45*, *51*) and DNA shuffling (*43*, *44*), respectively. These libraries were

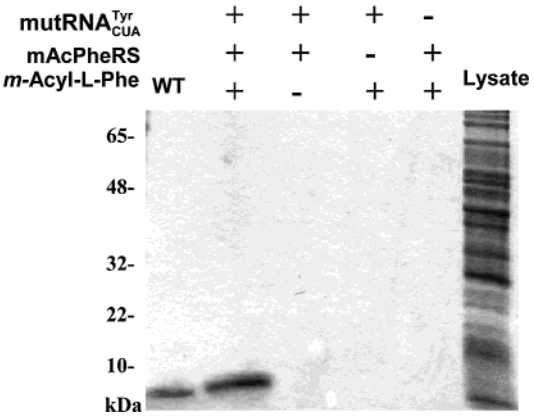


FIGURE 1: SDS−PAGE profile of expression of mutant Z domain proteins under various conditions. The gel was stained with Coomassie blue.

combined to afford approximately 1.5×10^8 different clones and were subjected to the same genetic selections again. Two TyrRS mutants were identified and isolated from this round of selection. Cells bearing these two mutant synthetase enzymes and mu tRNA$^{Tyr}_{CUA}$ survived in 120 μg/mL chloramphenicol in the presence of *m*-acetylphenylalanine and in 10 μg/mL chloramphenicol in the absence of the unnatural amino acid. These results suggest that the newly selected mutant TyrRS enzymes have improved selectivity for *m*-acetyl-L-phenylalanine relative to natural amino acids.

DNA sequencing revealed that these two candidate mutant synthetases had the same DNA sequence with the following mutations: Y32 → L, D158 → E, I159 → P, H160 → Q, Y161 → G, L162 → R, and G163 → D (Figure 1A). Structural models based on the crystal structure of the homologous TyrRS from *B. stearothermophilus* suggest that mutations of these seven amino acid residues may generate extra space in the active site of TyrRS to accommodate the acetyl group at the meta position of the phenyl ring and eliminate hydrogen bonds from residues Tyr32 and Asp158 to the tyrosine substrate. Among the mutations, several of them (from Glu158 to Asp163) are localized, suggesting the possibility that further mutation in this region may accommodate additional *meta*-substituted amino acids.

TyrRS mutants have been evolved to specifically charge *p*-acetylphenylalanine using the same genetic selection (*38*). Comparison of mutations between *p*-acetylphenylalanine-charging TyrRS enzymes and *m*-acetylphenylalanine-charging TyrRS enzyme shows that four residues (Tyr32, Asp158, Ile159, and Leu162) are mutated in both TyrRS mutants. Tyr32 was mutated to the hydrophobic Leu or Ala, and Leu162 was mutated to the hydrophilic Arg. The specificity of these mutant TyrRS enzymes toward keto-L-phenyalanine may result from the formation of a new hydrogen bond between Arg162 and the ketone group and loss of a hydrogen bond between the hydroxyl group of tyrosine and Tyr32. The crystal structures of the mutant TyrRS enzymes complexed with *p*-acetylphenylalanine and *m*-acetylphenylalanine are required to determine the exact roles of these mutations.

Incorporaton of m-Acetyl-L-phenylalanine into the Z Domain Protein. The mutant synthetase (termed mAcPheRS) was used to incorporate *m*-acetyl-L-phenylalanine in response to an amber codon at a permissive position (Lys7) of the Z domain protein. The Z domain is a small protein (~7.8 kDa)

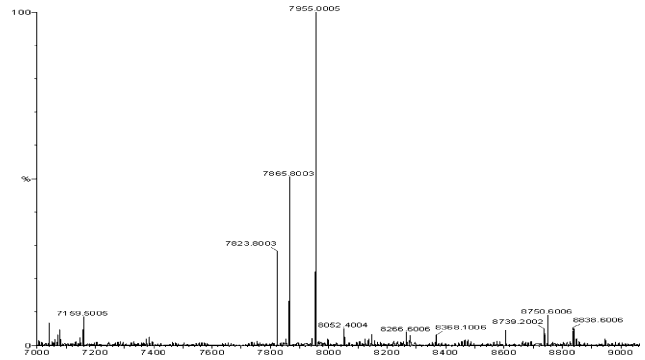


FIGURE 2: High-resolution mass spectrum (electrospray ionization) for the mutant Z domain containing *m*-acetyl-L-phenylalanine in place of Lys7. The average molecular mass of the intact keto Z domain protein was observed to be 7955.0005 Da. The molecular mass of the acetylated keto Z domain protein without the first methionine was observed to be 7865.8003 Da. The molecular mass of the keto Z domain protein without the first methionine was observed to be 7823.8003 Da.

and was chosen as a model protein to facilitate mass spectrometric analysis. Cells expressing mAcPheRS, mu tRNA$^{Tyr}_{CUA}$, and mutant Z domain protein were grown in the presence of *m*-acetylphenylalanine in GMML medium. As negative controls, the same cells were grown in the absence of either orthogonal tRNA, mAcPheRS, or *m*-acetylphenylalanine. The Z domain proteins in this experiment were purified by means of the C-terminal His6 tag. SDS−PAGE with Coomassie staining (Figure 1B) shows that the expression of the full-length Z domain protein is dependent upon the presence of *m*-acetylphenylalanine **2**, mu tRNA$^{Tyr}_{CUA}$, and mAcPheRS. No Z domain protein was observed in the absence of either this unnatural amino acid, mu tRNA$^{Tyr}_{CUA}$, or mAcPheRS (Figure 1B). This experiment suggests that the incorporation of *m*-acetyl-L-phenylalanine occurs with high fidelity at Lys7 in the Z domain protein. The yield of the expressed mutant Z domain protein containing *m*-acetylphenylalanine was approximately 1.8 mg/L, compared to 9.2 mg/L for the wild-type Z domain protein (*34*) and 3.6 mg/L for the *p*-acetylphenylalanine-mutant protein (*38*).

His6-purified mutant Z domain protein was further purified by reverse phase HPLC. Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) was used to measure the molecular mass of the purified mutant Z domain protein (Figure 2). There are three major peaks in the mass spectra corresponding to the intact protein, the protein without the first methionine, and the acetylated protein without the first methionine. The experimentally determined monoisotopic mass of the Z domain protein containing *m*-acetyl-L-phenylalanine is 7950.091 Da, which agrees within 27 ppm with the theoretical mass of 7949.874 Da. The experimental average mass for the intact protein is 7955.000 Da ($M_{theoretical}$ = 7954.727 Da). The experimental average mass for the mutant Z domain protein without methionine is 7823.800 Da ($M_{theoretical}$ = 7823.530 Da). The experimental average mass for the acetylated keto Z domain protein without methionine is 7865.800 Da ($M_{theoretical}$ = 7965.567 Da). The signal-to-noise ratio of more than 1000:1 suggests excellent fidelity for incorporation of *m*-acetyl-L-phenylalanine (>99%), similar to the level reported for *p*-acetyl-L-phenylalanine (*38*).

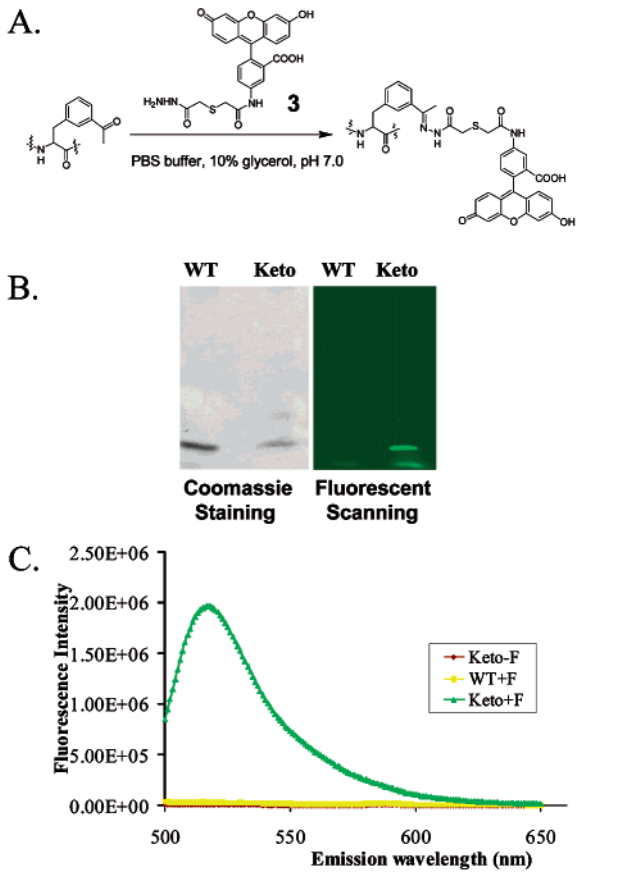


FIGURE 3: *In vitro* labeling of the mutant Z domain protein containing *m*-acetyl-L-phenylalanine with fluorescein hydrazide **3**. (A) Labeling reaction between *m*-acetyl-L-phenylalanine and fluorescein hydrazide **3**. (B) Coomassie-stained SDS−PAGE (left) profile and fluorescence image (right) of the wild-type Z domain protein (WT) and the keto mutant Z domain protein (Keto) treated with fluorescein hydrazide **3**. (C) Fluorescence spectra of *in vitro*-labeled Z domain protein mutants. "Keto-F" refers to the Z domain protein containing *m*-acetyl-L-phenylalanine only. "WT+F" refers to the wild-type Z domain protein labeled with fluorescein hydrazide. "Keto+F" refers to the Z domain protein containing *m*-acetyl-L-phenylalanine labeled with fluorescein hydrazide **3**.

In Vitro Labeling of Protein with Fluorescein Hydrazide. The purified Lys7 → *m*-acetylphenylalanine mutant Z domain protein was treated with fluorescein hydrazide **3** in aqueous phosphate buffer (1× PBS at pH 7.0) (Figure 3A). As a control, wild-type Z-domain protein was subjected to the same reaction conditions. After the reaction, both proteins were purified using a size exclusion PD-10 column to remove excess fluorescein hydrazide and analyzed with SDS−PAGE. The fluorescent signals from the gel were detected using a Storm imaging system (Figure 3B). The gel was then stained with Coomassie blue. The band for the mutant Z domain protein containing *m*-acetylphenylalanine was fluorescent, whereas no fluorescent signal was detected from the wild-type Z domain protein. Fluorescence emission spectra of these two labeled proteins were obtained by exciting each protein with 490 nm UV light (Figure 3C).

No fluorescence was detected from the unlabeled mutant Z domain protein (Keto-F) as well as the wild-type Z domain protein (WT+F), showing that fluorescein hydrazide does not react with any existing side chains from natural amino acids under these conditions. When the keto group was incorporated in Z domain protein, the protein was selectively labeled with fluorescein hydrazide. The labeled keto mutant Z domain protein was strongly fluorescent (Keto+F). The labeled and unlabeled mutant Z domain proteins were further separated by HPLC to examine the labeling efficiency. The ratio of the peak area of the labeled Z domain protein to total Z domain protein was 90 ± 5%.

The above *in vitro* protein labeling results are consistent with the data from the same *in vitro* labeling experiments that were performed on the mutant Z domain protein containing *p*-acetyl-L-phenylalanine. In fact, in addition to the high fidelity of the labeling reactions, the labeling efficiencies for both the Z domain protein containing *p*-acetyl-L-phenylalanine and the Z domain protein containing *m*-acetyl-L-phenylalanine are similar (90 ± 5%) (*38*).

In Vivo Modification of the Z Domain Protein with Hydrazide Derivatives. To determine whether site-specific protein modification with fluorescein hydrazide can be performed *in vivo*, *E. coli* cells expressing mutant Z domain protein containing *m*-acetylphenylalanine were washed extensively to remove the unnatural amino acid from the medium. The cells were then incubated with the membrane-permeable dye fluorescein hydrazide (1 mM, **3**, Figure 3A) dissolved in 1× PBS supplemented with 10% glycerol. After 12 h at 4 °C and extensive washing to remove excess fluorescein hydrazide, the labeled Z domain protein was purified via its C-terminal His6 tag using Ni^{2+}−NTA beads. As a negative control, wild-type Z domain protein was expressed, labeled *in vivo*, and purified using the same procedures. Fluorescence spectra were measured for both Z domain proteins with an excitation wavelength of 491 nm (Figure 4A).

The results show that only the mutant Z domain protein containing *m*-acetylphenylalanine was labeled with fluorescein hydrazide (Keto+F); no detectable fluorescence was observed with the wild-type Z-domain protein (WT+F). This result is consistent with the *in vitro* labeling data and demonstrates the specificity of ketone-mediated protein modification in an *in vivo* process. To determine the efficiency of *in vivo* labeling, the protein mixture purified with Ni^{2+}−NTA beads was further purified by a reverse phase HPLC. The ratio of peak area of the labeled Z domain protein to that of the total protein is ∼75%.

The HPLC-purified fluorescein-labeled Z domain protein was subjected to high-resolution MALDI (matrix-assisted laser desorption ionization) mass spectroscopy, and its correct molecular mass was determined to be 8424.113 Da ($M_{theoretical}$ = 8424.958 Da) (data not shown). This experiment confirms a molar ratio of 1:1 between the mutant Z domain and fluorescein hydrazide and demonstrates the excellent specificity of the labeling methodology.

To demonstrate the generality of this method, biotin hydrazide **4** was also used to label the mutant Z domain protein expressed *in vivo* (Figure 4B). The same labeling procedures and reaction conditions for labeling *in vivo* with fluorescein hydrazide were used. After the labeling reaction, cells were washed thoroughly to remove excess biotin hydrazide. Cells were lysed, and the full-length Z-domain protein was purified with the C-terminal His6 tag with Ni^{2+}−NTA beads. The purified protein was further purified by size exclusion chromatography to remove excess biotin hydrazide. The protein was then loaded onto an SDS−PAGE gel and transferred to a nitrocellulose membrane. The biotin-labeled

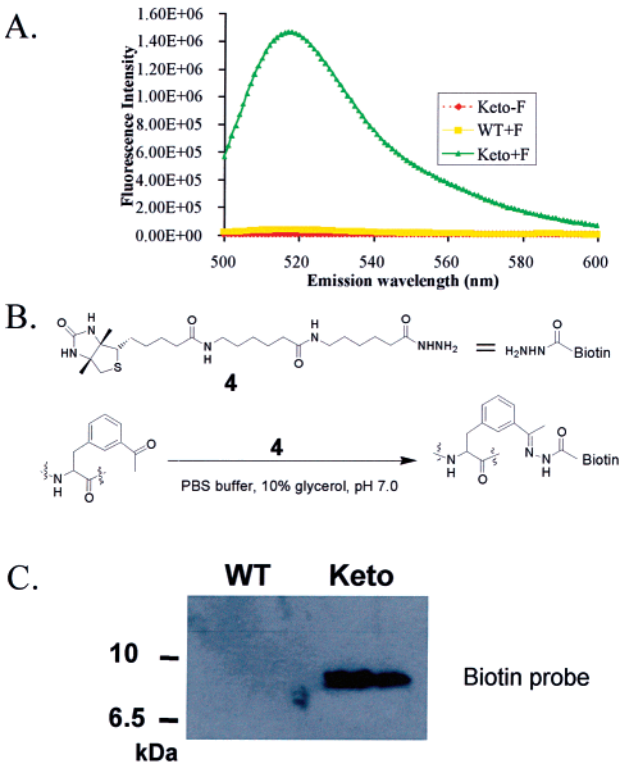


FIGURE 4: *In vivo* labeling of the mutant Z domain protein containing *m*-acetyl-L-phenylalanine with hydrazide derivatives. (A) Fluorescence spectra of the keto Z domain protein labeled with fluorescein hydrazide **3** *in vivo*. Refer to the legend of Figure 3 for the description. (B) Labeling reaction between incorporated *m*-acetyl-L-phenylalanine and biotin hydrazide **4**. (C) Western blot analysis of the endogenously expressed wild-type Z domain protein (WT) and the Z domain protein containing *m*-acetyl-L-phenylalanine (Keto) labeled with biotin hydrazide *in vivo*. Avidin-bound HRP was used to stain the biotin-labeled Z domain protein.

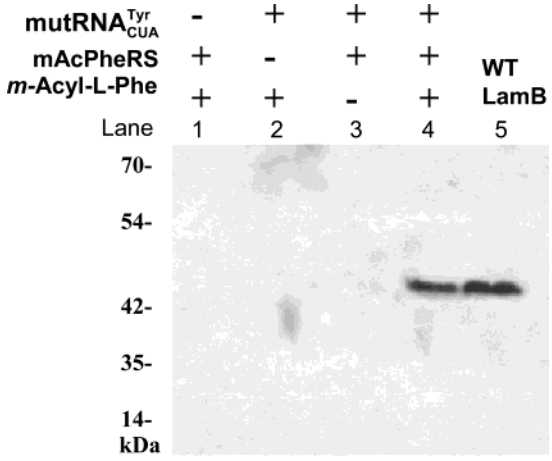


FIGURE 5: Western blot analysis of the expression of the LamB200Am protein under various conditions. Wild-type LamB and LamB200Am were stained with anti-LamB rabbit polyclonal antibody. Expression plasmids and amino acid supplements are indicated (lanes 1−4). LamB200Am was expressed in the presence of orthogonal tRNA, mAcPheRS, and *m*-acetylphenylalanine (lane 4). No LamB200Am was expressed in the absence of either orthogonal tRNA (lane 1), mAcPheRS (lane 2), or the unnatural amino acid (lane 3). Lane 5 shows a cell lysate containing the wild-type LamB protein.

Z domain protein was identified by staining with HRP-fused avidin (Figure 4C). As expected, only the mutant Z domain protein containing *m*-acetylphenylalanine was labeled with biotin hydrazide and detected with HRP-bound avidin. No signal was observed for the wild-type Z domain protein (Figure 4C).

The protein isolated from the Ni^{2+}−NTA beads was further purified by reverse phase HPLC as described above. The yield of *in vivo* protein labeling with biotin hydrazide was calculated by comparing the peak area between labeled and unlabeled Z domain proteins. The yield was approximately 75%, which is consistent with that for *in vivo* labeling with fluorescein hydrazide.

While this approach works well for labeling membrane-bound proteins *in vivo* (*vide infra*) and purified proteins *in vitro*, a high background was observed when labeling cytoplasmic proteins *in vivo*. Cells endogenously overexpressing the keto Z domain protein and expressing the wild-type Z domain protein were treated with membrane-permeable fluorescein hydrazide **3**. After being extensively washed, all cells were fluorescent, and the ratio of intensities between cells expressing the keto Z domain proteins and cells expressing the wild-type Z domain proteins (background) was roughly 10:1 (data not shown). The high background may be due to a high concentration of labeled ketone and aldehyde metabolites (*52*) as well as an inability to remove fluorescein hydrazide from the cells efficiently. We are currently exploring several approaches to overcoming this problem, including the use of fluorescence resonance energy transfer (FRET) (*53*−*55*) between the fluorescent group of the labeled keto mutant protein and a C- or N-terminally fused GFP. A similar approach could be used to probe interactions between a labeled fluorescent keto mutant protein and a second protein or ligand bearing an appropriate FRET donor or acceptor.

In Vivo Modification of LamB with Fluorescein Hydrazide Derivatives. To illustrate the utility of this methodology for selectively labeling a membrane protein in living cells, *m*-acetyl-L-phenylalanine was incorporated into the membrane protein LamB and then conjugated selectively with non-membrane-permeable dyes. LamB (maltoporin) is an outer membrane porin, whose biological functions include the transport of maltose and maltodextrins across the cell membrane (*56*−*59*). LamB also acts as the receptor for λ phage infection (*60*, *61*). This protein consists of 421 amino acids, and its X-ray structure reveals an 18-strand β-barrel structure (*58*). LamB was chosen as a model system for this study because its structure and activity are well-characterized (*62*).

Residue Asp200 of LamB is known to be a permissive site (*63*). The X-ray structure of the LamB protein reveals that residue Asp200 is located on a loop region exposed to the medium (*58*). A ketone group incorporated at position 200 of LamB should be accessible to hydrazide-derivatized fluorescent dyes added to the medium. Residue Asp200 was substituted with *m*-acetylphenylalanine **(**mAcPhe), and the resulting mutant LamB protein was expressed *in vivo* using the methodology described above. The host strain is GS20, an *E. coli* strain with a genomic deletion of the LamB gene. GS20 cells were transformed with a plasmid encoding the LamB gene with an amber codon at Asp200. The expression of wild-type and mutant LamB proteins was examined by SDS−PAGE of denatured bacterial extracts and immunoblotting with anti-LamB rabbit polyclonal serum (Figure 5).

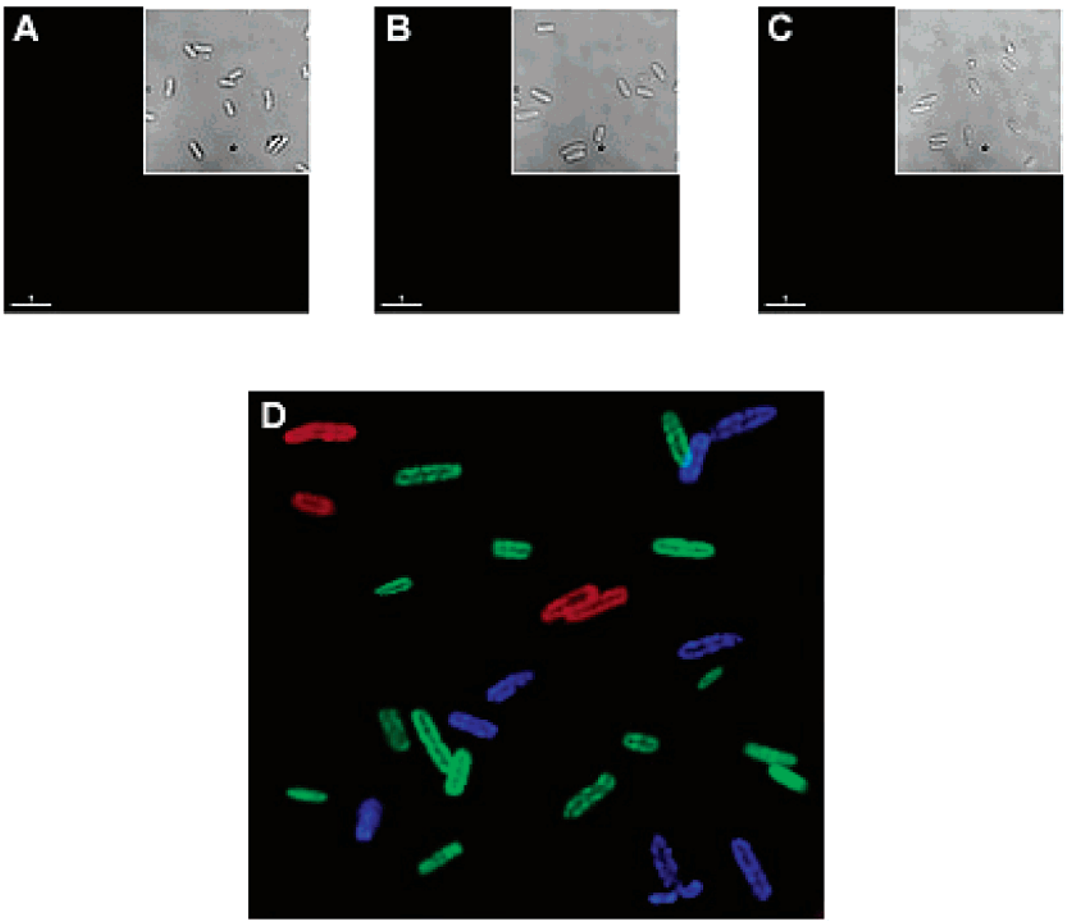


FIGURE 6: Fluorescence images of *E. coli* suface-expressed LamB mutant containing *m*-acetyl-L-phenylalanine that was labeled with non-membrane-permeable dyes. Panels A−C show the fluorescence imaging of GS20 cells grown in the absence of either orthogonal tRNA (A), mAcPheRS (B), or *m*-acetylphenylalanine (C) and labeled with non-membrane-permeable dyes. The insets show the bright field imaging of the corresponding GS20 cells. Panel D is the fluorescence imaging of the LamB200Am protein expressed in GS20 cells in the presence of orthogonal tRNA, mAcPheRS, and *m*-acetylphenylalanine and labeled with Cascade blue hydrazide (blue), Alexa568 hydrazide (red), and Alexa647 hydrazide (green). Note that there is virtually no background fluorescence from GS20 cells without expressed LamB200Am (A−C).

Under denaturing conditions, a single band (~46 kDa) was detected corresponding to the monomeric form of wild-type LamB. No expression of LamB was detected in the absence of either orthogonal tRNA, mACPheRS, or *m*-acetylphenylalanine. However, the full-length mutant LamB protein was expressed and detected as a single band when GS20 cells were grown in the presence of orthogonal tRNA, mACPheRS, and *m*-acetylphenylalanine. This *in vitro* biochemical data support the incorporation of *m*-acetylphenylalanine into LamB200mAcPhe *in vivo*.

The transport capacity of the mutated LamB was assayed *in vivo* under conditions which ensure that the LamB protein is limiting for transporting nutrient, i.e., at a maltose concentration of 1% in M9 minimal medium (*61*, *62*). The results are summarized in Table 1. GS20 cells that lack LamB do not survive in minimal medium containing maltose as a carbon source. In the absence of either the orthogonal tRNA, the orthogonal synthetase, or an unnatural amino acid, GS20 cells did not survive in minimal medium with 1% maltose, consistent with an inability to incorporate *m*-acetyl-L-phenylalanine and produce LamB protein. When the orthogonal tRNA, orthogonal synthetase, and unnatural amino acid are all present, GS20 cells survive in minimal medium with 1% maltose (Table 1), consistent with their ability to produce full-length mutant LamB (LamB200mAcPhe) *in vivo*. The data suggest that LamB200mAcPhe is functional and able to transport maltose into cells.

Table 1: Assays for Expression of Functional LamB200mAcPhe

cells	M9, leucine, and 10% maltose	phage titer (pfu)
Top 10	+	8.4×10^8
GS20	−	lawn
GS20 with WTLamB	+	1×10^8
GS20 with pmAcPhe, pLamB200mAcPhe, and **2**	−	lawn
GS20 with pYCJ17, pLamB200mAcPhe, and **2**	−	lawn
GS20 with pmAcPhe, pYCJ17, and pLamB200mAcPhe	−	lawn
GS20 with pmAcPhe, pYUCJ17, pLamB200mAcPhe, and **2**	+	1.5×10^7

The mutant LamB was also assayed for its ability to be recognized by λ phage (*64*); the results are shown in Table 1. GS20 cells that lack wild-type LamB are not superinfected by λ phage and form a lawn on GMML plates. With wild-type LamB expressed on the cell surface, these cells are superinfected with lytic λ phage and lysed. GS20 cells are

not able to produce full-length LamB in the absence of either mu $tRNA^{Tyr}_{CUA}$, mAcPheRS, or unnatural *m*-acetylphenylalanine. These LamB-deficient cells also survive the λ phage superinfection to form a lawn on GMML plates. GS20 cells that express LamB200mAcPhe in the presence of the mu $tRNA^{Tyr}_{CUA}$, mAcPheRS, and *m*-acetylphenylalanine are recognized, infected, and lysed by λ phage (1×10^7 pfu). This indicates that the expressed mutant LamB protein still serves as a functional λ phage receptor. These two different assays show that a point mutation at residue Asp200 of LamB with the unnatural *m*-acetylphenylalanine does not impair LamB protein function, either as a maltose transporter or as a λ phage receptor.

GS20 cells that express LamBmAcPhe were incubated separately with Cascade blue hydrazide, Alexa568 hydrazide, and Alexa647 hydrazide dissolved in 1× PBS with 10% glycerol overnight at 4 °C. The three dyes used in this experiment are not membrane-permeable. They can be easily removed by washing with aqueous buffer. After extensive washing to remove the nonspecific interaction of dyes with the cell membrane, these three types of labeled *E. coli* were mixed and immobilized on a 1.5% agarose slab. Images were captured on an Olympus IX-70 inverted microscope with a CCD camera. The mixed cells were excited with light at different wavelengths: 400 nm (Cascade blue), 576 nm (Alexa568), and 624 nm (Alexa 647). The cell images were recorded digitally at different emission wavelengths: 420 nm (Cascade blue), 599 nm (Alexa568), and 643 nm (Alexa647). The three images were merged to give the final three-color image in Figure 6. The blue cells are labeled with Cascade blue hydrazide, the red cells with Alexa568 hydrazide, and the green cells with Alexa647 hydrazide.

GS20 cells did not express LamB200mAcPhe in the absence of either mu $tRNA^{Tyr}_{CUA}$, mAcPheRS, or unnatural *m*-acetylphenylalanine. After the cells were treated with any of the three dyes, no fluorescence was visible after extensive washing (Figure 6A−C). In the presence of the orthogonal tRNA, the orthogonal synthetase, and the unnatural amino acid, mutant LamB was expressed on the GS20 cell surface. When these cells were treated with any of three dyes, the mutant LamB was labeled. The LamB200mAcPhe-expressing GS20 cells appeared as hollow cylinders after being labeled with either Cascade blue hydrazide, Alexa568 hydrazide, or Alexa647 hydrazide (Figure 6D), suggesting that only membrane-bound LamB protein was labeled. The conditions for the labeling reactions produced no morphological changes in the cells. These results suggest that one can selectively modify the cell surface by incorporating a ketone group into a membrane protein without affecting the biological function of the protein of interest. Such methodology should prove to be useful in examining conformational changes, localization, and protein−protein and protein−small molecule interactions involving membrane proteins.

CONCLUSION

In summary, we have site-specifically incorporated the unnatural amino acids *m*-acetyl-L-phenylalanine and *p*-acetyl-L-phenylalanine into proteins *in vivo* in response to the amber nonsense codon. Incorporation of these unnatural amino acids occurs with high fidelity and in good yields. The incorporated ketone group has been labeled selectively and efficiently with hydrazide derivatives both *in vitro* and *in vivo*. At present, the *in vivo* labeling methodology is particularly useful for the selective modification of cell surface proteins. This provides a powerful approach for cell membrane engineering and for the analysis of membrane protein structure and function. Other chemical groups may be site-specifically attached to proteins *in vivo* by the same ketone−hydrazide approach, including photoactive cross-linking reagents and mono- or oligosaccharides. *In vivo* fluorescence resonance energy transfer (FRET) studies should also be facilitated by the introduction of a ketone handle into proteins.

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