

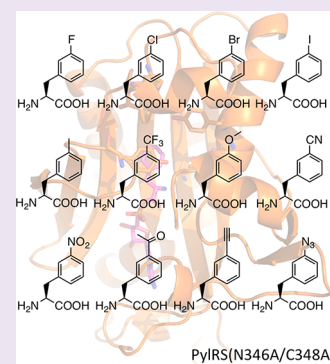
Genetic Incorporation of Twelve *meta*-Substituted Phenylalanine Derivatives Using a Single Pyrrolysyl-tRNA Synthetase Mutant

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S Supporting Information

ABSTRACT: When coexpressed with its cognate amber suppressing tRNA^{Pyl}_{CUA}, a pyrrolysyl-tRNA synthetase mutant N346A/C348A is able to genetically incorporate 12 *meta*-substituted phenylalanine derivatives into proteins site-specifically at amber mutation sites in *Escherichia coli*. These genetically encoded noncanonical amino acids resemble phenylalanine in size and contain diverse bioorthogonal functional groups such as halide, trifluoromethyl, nitrile, nitro, ketone, alkyne, and azide moieties. The genetic installation of these functional groups in proteins provides multiple ways to site-selectively label proteins with biophysical and biochemical probes for their functional investigations. We demonstrate that a genetically incorporated trifluoromethyl group can be used as a sensitive ¹⁹F NMR probe to study protein folding/unfolding, and that genetically incorporated reactive functional groups such as ketone, alkyne, and azide moieties can be applied to site-specifically label proteins with fluorescent probes. This critical discovery allows the synthesis of proteins with diverse bioorthogonal functional groups for a variety of basic studies and biotechnology development using a single recombinant expression system.



Site-selective modification is an important biotechnological strategy to introduce new functionalities to proteins. Examples include the PEGylation of therapeutic proteins to prolong their *in vivo* half-lives,¹ fluorescent labeling of proteins for protein folding/unfolding analysis,² linking proteins with photo-crosslinkers for protein–protein/DNA interaction studies,^{3,4} and introducing NMR, EPR, and IR probes for protein functional investigations.^{5–7} Traditionally, protein modifications use the high reactivity of cysteine and lysine side chains.^{8,9} However, this approach lacks selectivity. Modern techniques that attempt to achieve site-selective protein modification include the genetic noncanonical amino acid (NAA) incorporation,¹⁰ native chemical ligation, expression protein ligation,^{11,12} enzymatic and chemical modifications of peptide tags,^{13–15} and specific modifications of protein N- and C-termini.^{16,17} These techniques, in general, seek to introduce functional groups that do not exist in the 20 canonical amino acids (CAAs). Some of these functional groups can undergo bioorthogonal reactions for further modifications. Using orthogonal aminoacyl-tRNA synthetases (aaRSs) from different cell origins and their corresponding suppressing tRNAs, more than 70 NAAs have been genetically incorporated into proteins in *E. coli*, *Saccharomyces cerevisiae*, and mammalian cells.^{18–21} These NAAs contain most functional groups that are chemically stable in an aqueous environment. Genetic incorporation of these NAAs has been used to synthesize proteins containing biophysical probes for structural and functional investigations, as well as proteins with different biochemical functionalities.

Although powerful, the genetic NAA incorporation approach usually requires using a uniquely evolved aaRS for a specific NAA. aaRS-tRNA_{CUA} pairs with different origins might have to be used for different cell strains due to the nonorthogonal nature of some aaRS-tRNA_{CUA} pairs toward other endogenous aaRS-tRNA pairs in these hosts. A single aaRS that allows genetically encoding NAAs with diverse bioorthogonal functional groups in both prokaryotic and eukaryotic cells will relieve the burden of evolving aaRSs and searching for aaRS-tRNA_{CUA} pairs with different origins for use in different cell strains. It will also provide a single recombinant expression system for genetic incorporation of diverse NAAs so that parallel studies can be carried out rapidly. One system that meets this standard is the wild-type PylRS-tRNA^{Pyl}_{CUA} pair. It has been demonstrated that wild-type PylRS has a relatively broad substrate spectrum. However, all NAAs that are recognized by wild-type PylRS are much larger than the 20 CAAs.^{22–24} They are not optimal choices for protein modifications when it is necessary to minimize the extent of structural perturbations. In addition, NAAs with functional groups such as ketone, halide, nitro, and nitrile moieties have not been successfully incorporated into proteins using wild-type PylRS. Schultz and co-workers recently demonstrated that a mutant *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase (pCFRS)-tRNA^{Tyr}_{CUA} pair that was originally evolved for *para*-cyano-phenylalanine is able to mediate genetic incorporation of multiple *para*-

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Scheme 1

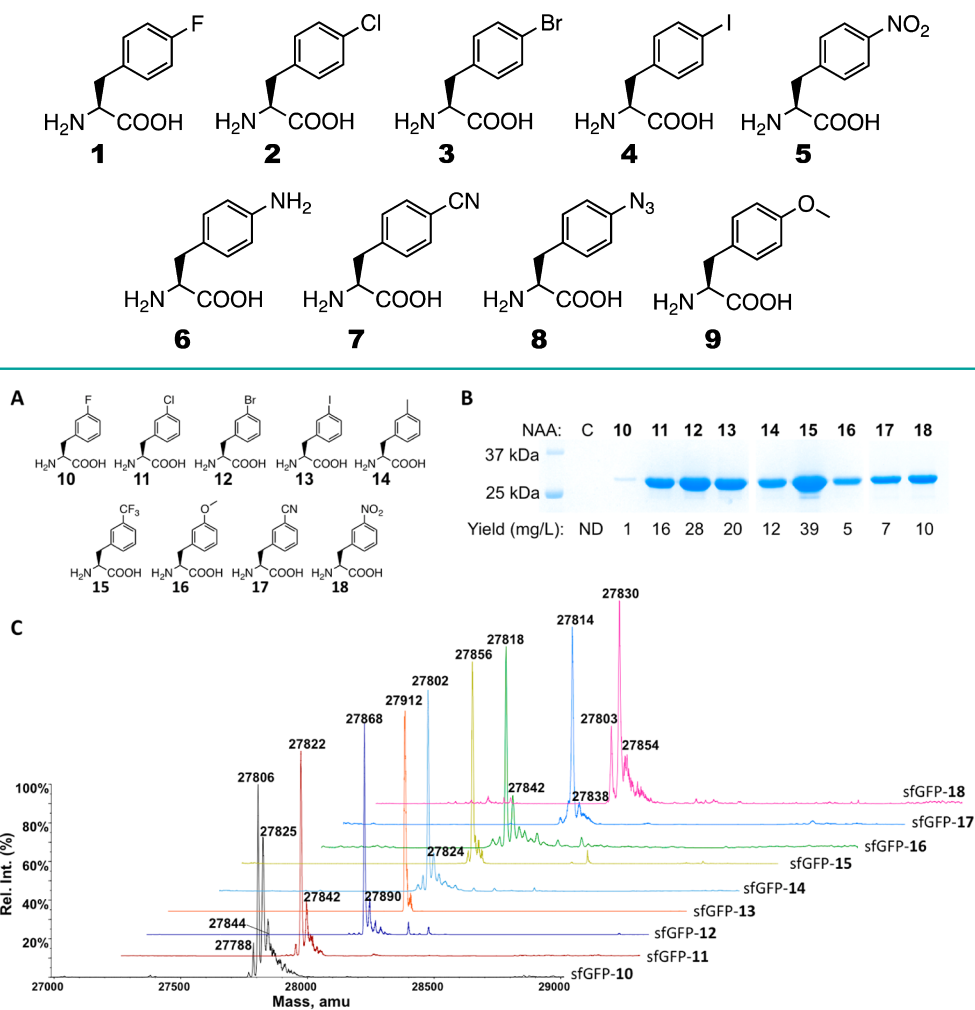


Figure 1. (A) Structures of NAAs 10–18. (B) Site-specific incorporation of 10–18 into sfGFP at its S2 position. (C) indicates a control experiment without the addition of any NAA. ND stands for nondetected. (C) Deconvoluted ESI-MS spectra of sfGFP variants incorporated with 10–18. In sfGFP-X, X is one of 10–18 incorporated at the S2 position.

substituted phenylalanine derivatives into proteins in *E. coli*.²⁵ However, this pair can only be used in bacteria due to its nonorthogonal nature of the pair in eukaryotic cells.²⁶

In one of our previous studies, we described a rationally designed PylRS mutant N346A/C348A.²⁷ When coexpressed with tRNA^{Pyl}_{CUA}, this enzyme mediates genetic incorporation of six tyrosine derivatives with large *O*-alkyl substituents into proteins at amber mutation sites in *E. coli*. Here, we reveal that the same enzyme-tRNA pair allows genetic incorporation of twelve phenylalanine derivatives with small *meta*-substituents and various bioorthogonal functional groups in *E. coli*. This new discovery allows the synthesis of diversely functionalized proteins using a single recombinant protein expression system in *E. coli*. Given the orthogonal nature of the PylRS-tRNA^{Pyl}_{CUA} pair in *S. cerevisiae*, *Caenorhabditis elegans*, and mammalian cells, the N346A/C348A-tRNA^{Pyl}_{CUA} pair can potentially be transferred into these cellular systems as well.^{28–32}

RESULTS

Genetic Incorporation of Nine *meta*-Substituted Phenylalanine Derivatives. In a previous study, we show that, in comparison to wild-type PylRS, N346A/C348A has an enhanced recognition of phenylalanine and mediates its

incorporation at an amber mutation site in *E. coli*.²⁷ *E. coli* BL21 cells transformed with two plasmids pEVOL-pylT-N346A/C348A and pEVOL-pylT-sfGFP2TAG that carry genes coding N346A/C348A, tRNA^{Pyl}_{CUA}, and superfolder green fluorescent protein (sfGFP) with an amber mutation at its S2 position were not able to express sfGFP in GMML (a minimal medium supplemented with 1% glycerol and 0.3 mM leucine). However, supplementing GMML with 2 mM phenylalanine induced sfGFP expression with a yield of 1.5 mg/L. When six tyrosine derivatives with large *O*-alkyl groups were used to supplement GMML to a final concentration of 2 mM, they all led to much higher sfGFP expression levels than that for phenylalanine, indicating that N346A/C348A might have higher binding affinities toward these NAAs. In contrast, when 2 mM phenylalanine derivatives with small *para*-substituents (2–9 shown in Scheme 1) were used to supplement GMML, sfGFP expression levels were much lower than for phenylalanine. Except for 9, the addition of all other NAAs led to negligible sfGFP expression levels. Following this previous study, recognition of 1 by N346A/C348A was recently tested. However, the same transformed *E. coli* BL21 cells could not grow at all in GMML supplemented with 2 mM 1. Apparently, 1 is highly toxic to *E. coli* BL21 cells.

Given that N346A/C348A has relatively high substrate promiscuity, four *meta*-halo-phenylalanine derivatives were also tested as substrates of N346A/C348A. Similar to the results shown in the previous study, *E. coli* BL21 cells transformed with pEVOL-pylT-N346A/C348A and pEVOL-pylT-sfGFP2TAG displayed undetectable sfGFP expression in GMML; however, providing 2 mM *meta*-chloro-phenylalanine, *meta*-bromophenylalanine, or *meta*-iodo-phenylalanine (**11**, **12**, and **13** in Figure 1A) in GMML induced sfGFP overexpression (Figure 1B). Expression levels under these conditions are all one order of magnitude higher than using 2 mM phenylalanine as a substrate of N346A/C348A. All three purified sfGFP variants had molecular weights determined by electrospray ionization mass spectrometry (ESI-MS) analysis that agreed well with their theoretical molecular weights (Figure 1C and Table 1).

Table 1. Calculated and Detected Molecular Weights of sfGFP Variants with Different NAAs Incorporated at S2

NAA	calculated mass (Da)	detected mass (Da) ^a
10	27806	27806, 27825, 27844
11	27822	27822
12	27867	27868
13	27914	27912
14	27802	27802
15	27856	27856
16	27818	27818
17	27812	27813
18	27829	27829
19	27830	27829
20	27813	27814
21	27833	27830

^aWith an error of ± 1 Da.

ESI-MS spectra of all three proteins also showed a small side peak corresponding to 20–24 Da more than the expected molecular weight. These small side peaks possibly originated from the sodium adduct ions that are commonly found in the ESI-MS analysis. Similar peaks were also found in other sfGFP proteins described later in this study. The uptake of *meta*-fluoro-phenylalanine (**10** in Figure 1A) by N346A/C348A was also tested. Although providing 2 mM **10** in GMML induced sfGFP expression, the yield was significantly lower than for **11**–**13**. Given that **10** could be misincorporated at phenylalanine sites, this low sfGFP expression yield might be due to a toxic effect of **10**.³³ Indeed, the harvested cell mass obtained from GMML supplemented with **10** was considerably less than from GMML supplemented with **11**–**13**. In addition, the ESI-MS spectrum of the purified sfGFP incorporated with **10** displayed several side peaks that indicate misincorporation of **10** at phenylalanine sites (Figure 1C and Table 1). Two mass peaks at 27 825 and 27 844 Da clearly indicate the presence of one and two additional residues of **10** at phenylalanine sites. A small peak at 27 788 Da matches the exact molecular weight of the sfGFP protein with a phenylalanine residue at the S2 amber mutation site. This indicates that **10** is not fully able to inhibit the binding of phenylalanine to N346A/C348A even though the recognition of phenylalanine by N346A/C348A is considered weak and that the concentration of phenylalanine in *E. coli* grown in minimal media is only about 20 μ M.³⁴ Mass peaks that indicate the incorporation of phenylalanine at S2 of sfGFP could not be identified in ESI-MS spectra of sfGFP proteins incorporated with other NAAs shown in this study.

Following the success with **11**–**13**, recognition of five other commercially available phenylalanine derivatives with small *meta*-substituents (**14**–**18** in Figure 1A) by N346A/C348A were tested. Growing *E. coli* BL21 cells transformed with pEVOL-pylT-N346A/C348A and pET-pylT-sfGFP2TAG in GMML supplemented with 2 mM **14**–**18** led to overexpression of sfGFP (Figure 1B). The major detected molecular weights of purified sfGFP variants determined by the ESI-MS analysis agreed well with their theoretic molecular weights (Figure 1C and Table 1). In most spectra, a minor mass peak that is at 20–24 Da higher than expected was detected. As discussed previously, these peaks are possibly from sodium adduct ions. The spectrum of sfGFP incorporated with **18** also displayed a minor peak at 27 803 Da. Although this peak matches the molecular weight of sfGFP incorporated with tyrosine at its S2 position (theoretical molecular weight: 27 804 Da), a tyrosine residue at S2 is unlikely. The previous study of N346A/C348A showed that this enzyme was not able to mediate genetic incorporation of tyrosine at an amber mutation site even when 2 mM tyrosine was provided in GMML.²⁷ Since *E. coli* contains a nitroreductase enzyme,³⁵ it is possible that **18** in sfGFP was reduced by this enzyme to *meta*-amino-phenylalanine (the theoretical molecular weight of sfGFP with *meta*-amino-phenylalanine incorporated at its S2 position is 27 802 Da). Compound **18** could also be reduced to *meta*-amino-phenylalanine that is then incorporated into sfGFP. To rule out this possibility, *E. coli* BL21 cells were grown in GMML supplemented with 2 mM *meta*-amino-phenylalanine. However, sfGFP was expressed at a very low and barely detectable level under this condition. Among all nine *meta*-substituted phenylalanine derivatives, **15** had the highest corresponding sfGFP expression yield. Among the NAAs that have been tested so far, **15** contains the most hydrophobic *meta*-substituent, a property that likely contributes to its strong interaction with the hydrophobic active site of N346A/C348A and therefore leads to its high incorporation rate. In comparison to all *O*-alkyl tyrosine derivatives and all *para*-substituted phenylalanine derivatives that were tested previously, most *meta*-substituted phenylalanine derivatives gave higher incorporation levels.

Using 15 As a Sensitive ¹⁹F NMR Probe to Study Protein Folding/Unfolding. Among **10**–**18**, **12**, and **13** could potentially undergo Suzuki–Miyaura cross-coupling reactions for site-selective modifications,³⁶ while **15** has three equivalent fluorine atoms that can serve as a sensitive ¹⁹F NMR probe for protein folding/unfolding and structural dynamics analysis,³⁷ the nitrile group in **17** is a strong IR probe,⁷ and **18** can be used as a distance probe due to its ability to quench the intrinsic fluorescence of a tryptophan residue in a protein.³⁸ In this study, we chose to demonstrate the application of **15** in protein folding/unfolding analysis. Mehl and co-workers previously showed that *para*-trifluoromethyl-phenylalanine could be genetically incorporated into enzymes in *E. coli* using an evolved *M. jannaschii* tyrosyl-tRNA synthetase (MjTyrRS)-tRNA^{Tyr}_{CUA} pair, and that genetically incorporated *para*-trifluoromethyl-phenylalanine residues in enzymes could be used as ¹⁹F NMR probes to sense the binding of substrates, inhibitors, and cofactors.³⁷ The same technique was also extended to side chain relaxation analysis of a SH3 domain.³⁹ In these studies, *para*-trifluoromethyl-phenylalanine was incorporated at the protein surface, which potentially avoided disruption of protein folding due to the steric hindrance that might be introduced by the incorporated *para*-trifluoromethyl-phenylalanine residue. Folding disruption might be a concern

when *para*-trifluoromethyl-phenylalanine is incorporated in a protein interior. Given that **15** is an isomer of *para*-trifluoromethyl-phenylalanine, it is a useful alternative of *para*-trifluoromethyl-phenylalanine for protein folding/unfolding and structural dynamics analysis and might be a solution when the incorporation of *para*-trifluoromethyl-phenylalanine into a protein interior disrupts folding. In the following experiments, **15** was genetically incorporated into sfGFP at both its surface and its interior. The resulting proteins were used to undergo unfolding analysis using ^{19}F NMR.

To express sfGFP incorporated with **15** at F27 (sfGFP27-**15**), a plasmid pBAD-sfGFP27TAG that carried a sfGFP gene with an amber mutation at F27 was constructed. F27 is located at the second β -strand of sfGFP. Its side chain faces toward the protein interior and is surrounded by several hydrophobic residues (Supplementary Figure 1). After unfolding, sfGFP is expected to completely expose F27 to a hydrophilic environment. We anticipated that **15** at F27 of sfGFP would show a large chemical shift change during the unfolding process. Together with pEVOL-pylT-N346A/C348A, pBAD-sfGFP27TAG was used to transform *E. coli* Top10 cells. The transformed cells were then grown in lysogenic broth (LB) media supplemented with 1 mM **15** and induced with the addition of 0.2% arabinose. This procedure resulted in sfGFP27-**15** expression at an expression level of 79 mg/L (Supplementary Figure 2). Only a minimal amount of sfGFP was expressed in LB without a NAA supplement. The purified sfGFP27-**15** had a fluorescent spectrum and intensity similar to wild-type sfGFP, and a sample of ^{15}N -sfGFP-**15** also showed a similar, but not identical, fingerprint as wild-type sfGFP in a [^{15}N , ^1H] correlation spectrum (Supplementary Figures 3 and 4).⁴⁰ Differences included small shifts in various peaks such as, e.g., those near 10 ppm/112 ppm and the appearance of a weak peak near 10.2 ppm/128 ppm. The relatively close agreement of the spectra suggests that replacing F27 with **15** does not significantly affect the protein folding and chromophore formation processes.

Purified sfGFP27-**15** was then used to undergo unfolding analysis in the presence of guanidinium chloride (GndCl). In the titration of sfGFP27-**15** against GndCl, ^{19}F NMR signals of sfGFP27-**15** were detected (Figure 2A). At low denaturant concentration, the chemical shift of folded sfGFP27-**15** was dependent on the GndCl concentration. This concentration dependence may likely be explained by a local structural change prior to denaturation. The appearance of the peak at 61.5 ppm indicates that the unfolding transition in this titration occurred at around 4.6 M GndCl, which, based on the time of 1 h between titrated points, appears consistent with an unfolding hysteresis observed in ref 40. Likewise, a stepwise titration of decreasing GndCl indicates the presence of unfolded sfGFP27-**15** to a much lower GndCl concentration of 1.6 M (Figure 2B). Similarly, sfGFP proteins with **15** incorporated at S2, F8, F130, and N135 positions were recombinantly expressed (Supplementary Figure 2) and used to undergo unfolding studies in the presence of GndCl and determined by ^{19}F NMR (Figure 2C). The locations of the mutations have been chosen such that S2 and F8 are at the beginning and in the first α -helix of the protein (F27 is in a β -sheet), and F130 and N135 are located in a loop, with the latter more exposed to the solvent than the former. All proteins were expressed as soluble and folded forms. In all mutants, the titration resulted in the appearance of a second peak corresponding to the unfolded form at intermediate concentrations of GndCl. Mutations located in

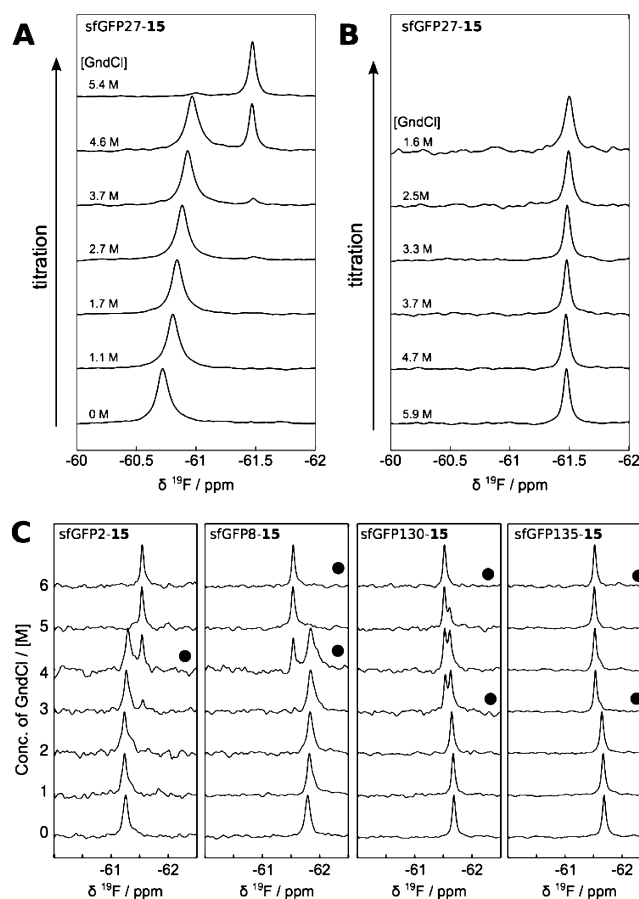


Figure 2. (A) Unfolding of sfGFP27-**15**, by stepwise addition of GndCl, characterized by ^{19}F NMR. Titrations were carried out by sequential addition of GndCl, starting from 0 M. (B) Spectra of unfolded sfGFP27-**15** upon stepwise reduction of GndCl. Precipitation occurred below 1.6 M GndCl. In panels A and B, time between each point was 1 h, and measurement temperature was 25 °C. (C) Unfolding of sfGFP2-**15**, sfGFP8-**15**, sfGFP130-**15**, and sfGFP135-**15** by stepwise addition of GndCl, characterized by ^{19}F NMR. Time between spectra was 2–3 h, except for the spectra indicated with (●), where additional time of 1–2 days was allowed for transitions to occur.

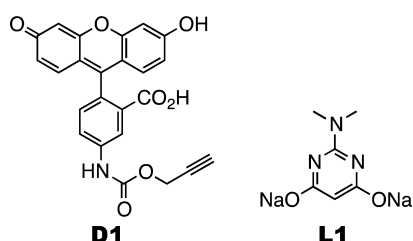
well-defined secondary structures of sfGFP, including those at F27, S2, and F8, show larger chemical shift differences than those located in loop regions. Further, a gradual chemical shift change, in some cases concomitant with line broadening, can be observed prior to denaturation. This change is also most prominent in the F8 and F27 mutants, which are located in well-structured regions. We also carried out fluorescence equilibrium unfolding analysis for all synthesized **15**-containing sfGFP proteins. Except sfGFP8-**15**, all other **15**-containing sfGFP proteins displayed unfolding patterns very similar to sfGFP proteins with phenylalanine at **15**-corresponding sites (Supplementary Figures 5–9).

The significant change in ^{19}F chemical shifts observed in this study illustrates that **15** can be used as a sensitive site-specific probe for protein folding or potentially for protein–ligand interaction. Since ^{19}F allows for highly receptive, background-free NMR detection, and the chemical shift of this nucleus is sensitive to structural changes,⁴¹ **15** inserted at a known position will be particularly useful for measurements with large proteins, or with dilute samples, where chemical shift assignments typically cannot be obtained. The present study also indicates that **15** resembles phenylalanine in size and its

incorporation into a protein may not disrupt its folding, although a careful analysis of this property for any particular protein incorporated with **15** is strongly recommended.

Genetic Incorporation of Three Chemically Reactive *meta*-Substituted Phenylalanine Derivatives for Site-Selective Protein Modifications. Since we previously showed that a *para*-iodo-phenylalanine residue that was incorporated into a protein using an evolved PylRS-tRNA^{Pyl}_{CUA} pair could be fluorescently labeled using a Suzuki–Miyaura cross-coupling reaction,⁴² a similar reaction to label **13** is not repeated here. Instead, we carried out a recently developed copper-free Sonogashira cross-coupling reaction⁴³ to label **13**. Both dye **D1** and a palladium ligand **L1** shown in Scheme 2

Scheme 2



were synthesized according to literature procedures.⁴³ Incubating sfGFP with **13** incorporated at S2 (sfGFP-**13**) together with dye **D1** in the presence of the **L1**-palladium(II) complex and sodium ascorbate at pH 7 for 3 h led to no fluorescein-labeled sfGFP-**13** that could be detected on a denaturing SDS-PAGE gel. To avoid the background fluorescent emission of sfGFP

itself, the sample was treated with 10% SDS and boiled for 20 min to totally denature sfGFP before it was analyzed. The same treatment was carried out with all other sfGFP variants that were labeled with small molecule fluorescein dyes, as described later. Prolonging the time of the reaction between sfGFP-**13** and **D1** to overnight induced a large amount of protein aggregation but no apparently improved fluorescein labeling (data not shown). Since the **L1**-palladium(II) complex needs to react with **13** to form an intermediate for a further reaction with **D1**,⁴³ we think the low concentration of sfGFP-**13** makes its reaction with **D1** kinetically unfavorable. The protein aggregation might be due to the interaction between exposed sulfur-containing cysteine and methionine residues of sfGFP-**13** and the **L1**-palladium(II) complex.

In order to synthesize proteins incorporated with chemically active *meta*-substituted phenylalanine derivatives for their site-selective modifications, we synthesized three NAAs shown as **19–21** in Figure 3A and tested their recognition by N346A/C348A. These NAAs were synthesized as racemic mixtures and used directly in the following analysis without further purification. Specific recognition of L-amino acids by aminoacyl-tRNA synthetases and the ribosome has been demonstrated elsewhere.^{44,45} *E. coli* BL21 cells transformed with pEVOL-pylT-N346A/C348A and pET-pylT-sfGFP2TAG were used to test **19–21**. The transformed cells displayed undetectable sfGFP expression in GMML; however, providing **19**, **20**, or **21** in GMML induced sfGFP overexpression (Figure 3B). Since **19–21** are racemic mixtures, 4 mM was added to yield a final concentration of 2 mM for the L-isomer. Expression levels under these conditions are much higher

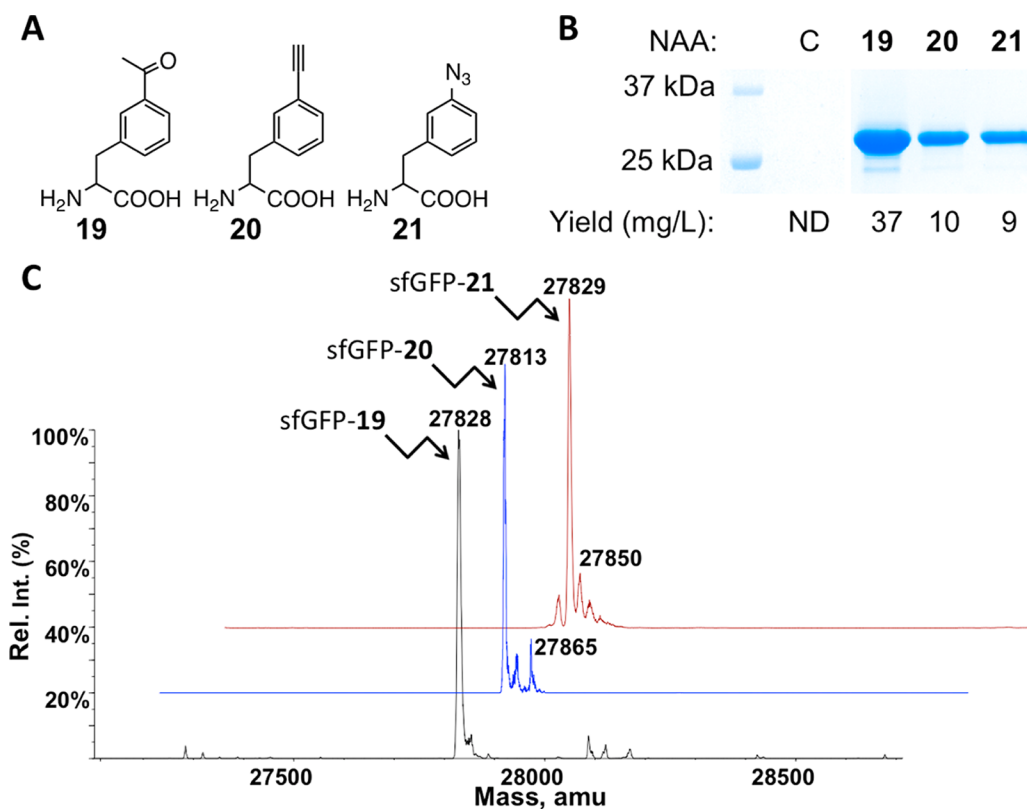


Figure 3. (A) Structures of **19–21**. (B) Site-specific incorporation of **19–21** into sfGFP at its S2 position. C indicates a control experiment without the addition of any NAA. ND stands for nondetected. (C) Deconvoluted ESI-MS spectra of sfGFP variants incorporated with **19–21**. In sfGFP-X, X is one of **19–21** incorporated at the S2 position.

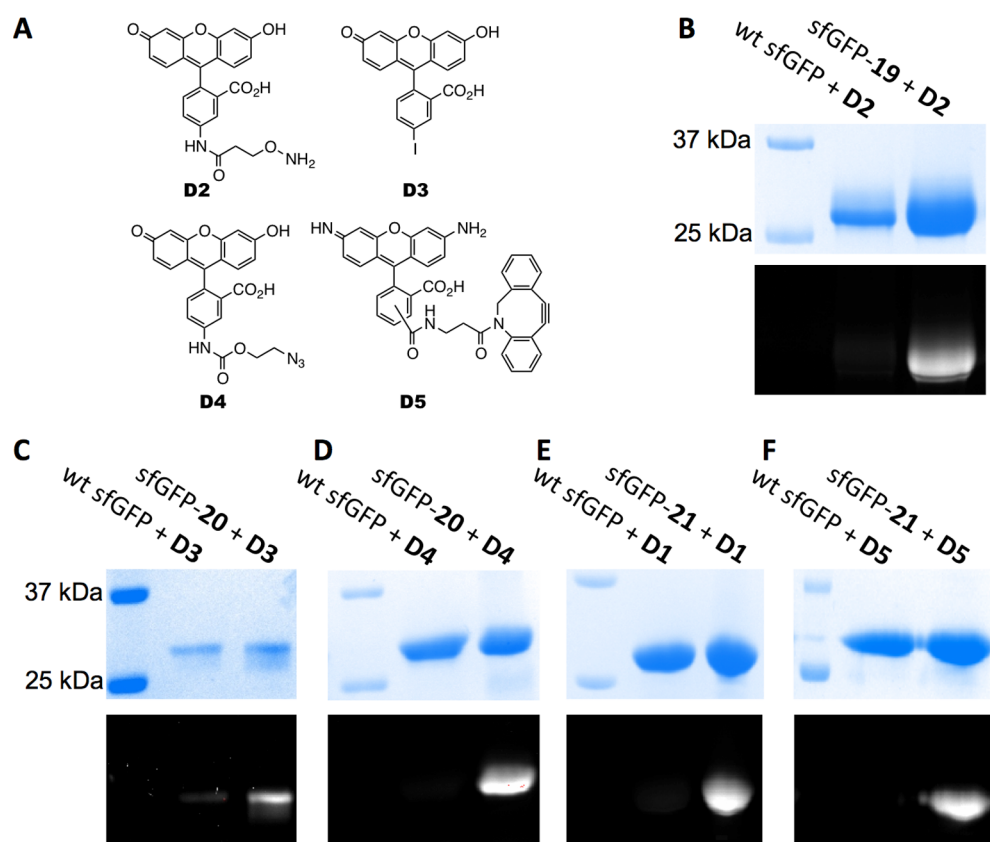


Figure 4. (A) Structures of dyes **D2–D5**. (B) Specific labeling of sfGFP-19 with **D2**. (C) Specific labeling of sfGFP-20 with **D3**. (D) Specific labeling of sfGFP-20 with **D4**. (E) Specific labeling of sfGFP-21 with **D1**. (F) Specific labeling of sfGFP-21 with **D5**. In panels B–F, the top panels show denaturing SDS-PAGE analysis of sfGFP proteins with Coomassie blue staining, and the bottom panels are from fluorescent imaging of the same gels before Coomassie blue staining. Wt sfGFP stands for wild-type sfGFP.

than using 2 mM phenylalanine as a substrate of N346A/C348A. All three purified sfGFP variants had molecular weights determined by ESI-MS that agreed well with their theoretical molecular weights (Figure 3C and Table 1). The sfGFP variant with **20** incorporated at S2 (sfGFP-20) also showed a minor mass peak at 27 865 Da. This peak is possibly from sfGFP-20 with *N*-terminal acetylation.⁴⁶ A minor peak at 27 850 Da was also found in the spectrum of sfGFP with **21** incorporated at S2 (sfGFP-21). As discussed previously, this peak is possibly from the sodium adduct ion of sfGFP-21.

With the sfGFP protein with **19** incorporated at S2 (sfGFP-19), sfGFP-20, and sfGFP-21 in hand, we then proceeded to their labeling with corresponding fluorescein dyes. Besides using the copper-free Sonogashira cross-coupling reaction to label sfGFP-21, the oxime formation reaction to label sfGFP-19 and the alkyne–azide Huisgen cycloaddition reaction^{47,48} to label sfGFP-20 and sfGFP-21 were also tested. Four additional fluorescein dyes **D2–D5** (Figure 4A) were either synthesized or purchased.^{43,49} As expected, sfGFP-19 reacted specifically with dye **D2** at pH 4 to give a fluorescein-labeled sfGFP-19 that showed intense fluorescence in a denaturing SDS-PAGE gel under UV irradiation (Figure 4B). As a control, wild-type sfGFP could not be labeled with **D2** under the same condition. We also tested the reaction of sfGFP-19 with **D2** at pH 7. An overnight incubation gave a negligible fluorescein-labeled sfGFP-19 (data not shown). This is consistent with what was described previously by Brustad et al.⁵⁰ Two reactions, the copper-free Sonogashira cross-coupling reaction and the alkyne–alkyne Huisgen cycloaddition reaction, were employed to label

sfGFP-20. The 3 h reaction between sfGFP-20 and dye **D3** in the presence of the L1-palladium(II) complex and sodium ascorbate at pH 7 resulted in a detectable fluorescein-labeled protein (Figure 4C). To achieve this labeling, 10 equiv of the catalyst was preincubated with 10 equiv of **D3** for 1 h to obtain an activated reagent cocktail. The final labeling reaction was performed with the addition of another 90 equiv of **D3** and 1 equiv of sfGFP-20 to this cocktail. Although we have obtained labeling of sfGFP-20 with **D3**, we did notice that a large portion of sfGFP-20 underwent aggregation during the labeling process. There was a relatively low level of background labeling of wild-type sfGFP with **D3** (Figure 4C). Labeling of sfGFP-20 with dye **D4** was carried out under an optimized condition that contained 0.1 mM Cu(I):tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) complex, 0.4 mM additional TBTA, 5 mM sodium ascorbate, and 1 mM NiCl₂ for 3 h.⁵¹ The finally labeled protein on a denaturing SDS-PAGE gel was intensely fluorescent under UV irradiation (Figure 4D). This same click labeling reaction was also used to label sfGFP-21 specifically with **D1** (Figure 4E). We also carried out the copper-free cyclooctyne–azide cycloaddition reaction to label sfGFP-21 with **D5**. An overnight direct incubation of sfGFP-21 with **D5** led to a fluorescein-labeled protein that showed strong fluorescence under UV irradiation. For all three click conditions, similar reactions with wild-type sfGFP were used as controls that did not give any detectable fluorescein-labeled final products. These assembled data demonstrate that diverse bioorthogonal groups installed in proteins using the N346A/C348A-tRNA^{Pyl}_{CUA} pair enable a variety of ways for site-specific protein modifications.

DISCUSSION

Genetic Incorporation of *meta*-Substituted Phenylalanine Derivatives. Using evolved MjTyrRS-tRNA^{Tyr}_{CUA} pairs, Schultz and co-workers showed that more than 20 *para*-substituted phenylalanine derivatives could be genetically incorporated into proteins at amber mutation sites in *E. coli*.^{7,18,38,50,52–68} Compared to *para*-substituted phenylalanine derivatives, genetic incorporation of *meta*-substituted phenylalanine derivatives is far less explored. Zhang et al. demonstrated that a *meta*-substituted phenylalanine derivative, **19**, could be genetically encoded in *E. coli* using an evolved MjTyrRS-tRNA^{Tyr}_{CUA} pair.⁶⁹ However, there has been no follow-up study since this work was originally published in 2003. Developing methods for genetic incorporation of other *meta*-substituted phenylalanine derivatives will not only expand the genetically encoded NAA inventory, leaving more choices for protein engineering when subtle variations are necessary but also help understand the scope of NAA tolerance of the cellular protein translation machinery. In this study, all twelve *meta*-substituted phenylalanine derivatives (**10–21**) have small substituents. Their incorporation into protein interiors is expected not to significantly disturb protein folding. This may allow using these NAAs, e.g., **15** and **17**, to probe local environments in the interior of proteins. Although *para*-substituted phenylalanine derivatives with similar functional groups have been incorporated into proteins in *E. coli* using multiple evolved MjTyrRS-tRNA^{Tyr}_{CUA} pairs, **10–21** may be excellent alternatives when the incorporation of *para*-substituted phenylalanine derivatives disturbs protein folding. There is also an advantage of using the N346A/C348A-tRNA^{Pyl}_{CUA} pair. This pair was derived from the wild-type PylRS-tRNA^{Pyl}_{CUA} pair that is orthogonal in *E. coli*, *S. cerevisiae*, and mammalian cells. The N346A/C348A-tRNA^{Pyl}_{CUA} pair can be potentially transferred into eukaryotic cell systems for the expression of proteins with diverse functional groups that cannot be achieved in *E. coli* due to protein folding problems or the need of post-translational modifications. So far, evolved MjTyrRS-tRNA^{Tyr}_{CUA} pairs can only be used in *E. coli* due to recognition of tRNA^{Tyr}_{CUA} by endogenous yeast and mammalian aaRSs (Liu and Schultz, unpublished data). Since genetic incorporation of NAAs has been used in phage display,⁷⁰ the current development also provides more chemical moieties that can be loaded into phage-displayed libraries to expand the structural diversity for drug discovery.

Recognition of *meta*-Substituted Phenylalanine Derivatives by N346A/C348A. In the previous study, we show that N346A/C348A is able to charge tRNA^{Pyl}_{CUA} with phenylalanine for genetic incorporation of phenylalanine at an amber stop codon in *E. coli*.²⁷ The phenylalanine recognition of N346A/C348A is probably due to the removal of the N346 amide that can potentially prevent the binding of phenylalanine to the active site.^{27,42} Since there is a large hydrophobic pocket at the active site of N346A/C348A, we predicted and demonstrated that tyrosine derivatives with large *O*-alkyl substituents could be recognized by N346A/C348A and genetically encoded in *E. coli* using the N346A/C348A-tRNA^{Pyl}_{CUA} pair. However, phenylalanine derivatives with small *para*-substituents could not be incorporated into proteins using the same system. Figure 5 presents the superimposed active site structures of two PylRS variants. One is wild-type PylRS with pyrrolysyl-AMP binding at the active site, and the second one is a mutant PylRS (OmeRS) complex with **9**.^{71,72} OmeRS was

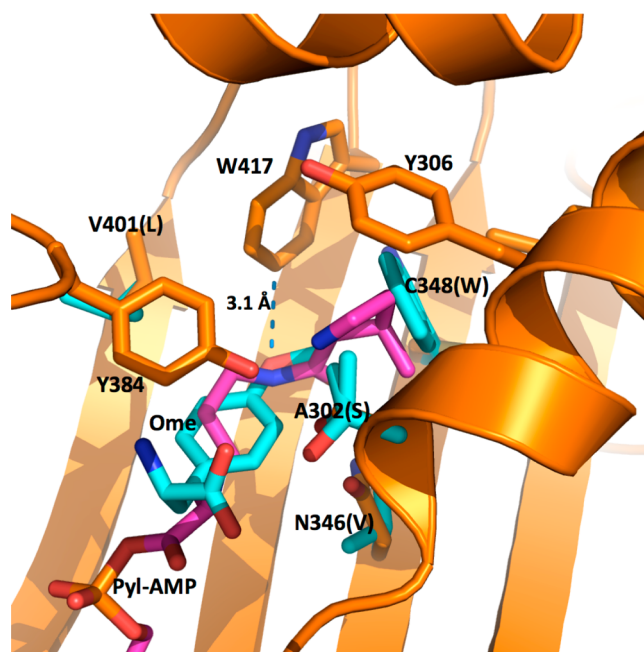


Figure 5. Superimposed structures of the PylRS complex with pyrrolysyl-AMP (Pyl-AMP) and the OmeRS complex with **9** (Ome). The PylRS complex with pyrrolysyl-AMP is shown in orange for the protein carbon atoms and pink for the pyrrolysyl-AMP carbon atoms. Four mutated residues in OmeRS and the ligand **9** are shown in cyan for the carbon atoms. Letters in the parentheses indicate four mutated residues in OmeRS.

specifically evolved for **9** by Wang and co-workers. Although Y384 is not observed in the crystal structure of the OmeRS complex with **9**, it is expected to locate at the top of **9** in a similar way as in the wild-type PylRS complex with pyrrolysyl-AMP. Yokoyama and co-workers have demonstrated that Y384 is critical for the aminoacylation of tRNA^{Pyl}_{CUA}, and multiple structures of PylRS have shown that Y384 is at the same position.^{23,73,74} With Y384 at its top, the aromatic side chain of **9** might have strong π - π stacking interactions with the side chain of Y384 and the two backbone amides of residues 419–421 (not shown in Figure 5). The methoxy oxygen atom of **9** is very close to W417 with a distance of 3.1 Å. It is possible that all *para*- and *meta*-substituted phenylalanine derivatives have a similar binding pattern when they interact with N346A/C348A. When a phenylalanine derivative has a small substituent such as azide, halide, nitrile, and nitro moieties, this small substituent that is still considerably larger than an oxygen atom might have a strong steric clash with W417 so that its binding to the active site of N346A/C348A is disfavored. Compound **9** has a flexible *O*-methyl group that might be located in the deep pocket of N346A/C348A for a favorable interaction. Tyrosine derivatives with larger *O*-alkyl groups that can potentially interact with the deep pocket with strong hydrophobic interactions might have more favorable interactions with N346A/C348A. The structure of OmeRS also shows that two active site residues at 346 and 348 are very close to the *meta*-position of the phenyl ring of **9**. Since these two residues are mutated to alanine residues in N346A/C348A, it is possible that a big hydrophobic pocket forms around the side-chain *meta*-position of phenylalanine when it binds to N346A/C348A. This hydrophobic pocket might contribute to favorable interactions between N346A/C348A and phenylalanine derivatives with small hydrophobic *meta*-substituents. The incorporation level of one particular

phenylalanine derivative is related to the aminoacyl-tRNA synthesis step, the binding of aminoacyl-tRNA to elongation factor Tu, the association of aminoacyl-tRNA to the ribosome, and the final peptidyl transfer reaction. Therefore, there may not be a direct correlation between the incorporation level of a NAA and its binding affinity to N346A/C348A. Assays are necessary to characterize the binding of phenylalanine derivatives to N346A/C348A and their aminoacylation reactions with tRNA^{Pyl}_{CUA}. In addition, the crystal structures of N346A/C348A complexes with different phenylalanine derivatives will provide additional structural insights. These are directions we intend to pursue in the future.

CONCLUSIONS

In summary, we demonstrated genetic incorporation of twelve *meta*-substituted phenylalanine derivatives with diverse bio-orthogonal functional groups into proteins in *E. coli* using a single PylRS mutant N346A/C348A together with its cognate amber suppressing tRNA^{Pyl}_{CUA}. This critical development makes it possible to use a single recombinant expression system to synthesize proteins with diverse chemical moieties for parallel studies. Among these twelve NAAs, **15** can be used as a sensitive ¹⁹F NMR probe, **17** can potentially serve as an IR probe,⁷ and **18** can be potentially used as a distance probe through its ability to quench the intrinsic fluorescence of tryptophan residues in proteins, and **13**, **19**, **20**, and **21** can undergo various bioorthogonal reactions for site-specific protein labeling with different biophysical and biochemical probes (see Supplementary Table 1 for other potential applications).³⁸ Except for **19** that was previously incorporated into proteins using an evolved *M. jannaschii* TyrRS-tRNA^{Tyr}_{CUA} pair, genetic incorporation of all other *meta*-substituted phenylalanine derivatives shown in this study is reported for the first time. In addition, all twelve *meta*-substituted phenylalanine derivatives resemble phenylalanine in size. The incorporation of these *meta*-substituted phenylalanine derivatives into proteins even in the protein interiors may not significantly disrupt protein folding as we demonstrated in the sfGFP unfolding studies using a genetically incorporated **15**. Since the wild-type PylRS-tRNA^{Pyl}_{CUA} pair has been used in *S. cerevisiae* and mammalian cells for NAA incorporation, it is expected that the N346A/C348A-tRNA^{Pyl}_{CUA} pair can be simply transferred to these cellular systems, potentially allowing various bioconjugation reactions to be carried out in these cells. We are currently engaged in transferring the system to eukaryotic cells and the progress will be reported later on.

METHODS

NAAs, Ligand L1, and Dyes. NAAs **1–18** were purchased from Chem Impex Inc. Dye **D5** was obtained from Click Chemistry Tools Inc. Ligand **L1** and dyes **D1–D4** were synthesized according to literature procedures.^{45,51,75} The synthesis of NAAs **19–21** is described in detail in the Supporting Information.

Plasmid Construction. Construction of pEVOL-pylT-N346A/C348A and pET-sfGFP2TAG was described previously.²⁷ Plasmid pBAD-sfGFP135TAG that carries a sfGFP gene with an amber mutation at its 135 position was a gift from Dr. Ryan Mehl at Oregon State University. Plasmids pBAD-sfGFP8TAG, pBAD-sfGFP27TAG, and pBAD-sfGFP130TAG that carry sfGFP genes with amber mutations at positions 8, 27, and 130, respectively, were derived from plasmid pBAD-sfGFP, a gift also from Dr. Ryan Mehl. Construction of these plasmids was carried out using a site-directed mutagenesis protocol that was based on Phusion DNA polymerase. In brief, two oligonucleotide primers, one of which covers the mutation

site, were used to amplify the whole plasmid of pBAD-sfGFP to give a blunt-end PCR product. This PCR product was phosphorylated by T4 polynucleotide kinase and then underwent self-ligation using T4 DNA ligase. Primers pBAD-sfGFP-F8TAG-F (5'-actgtgtgttctctattcttctgtgaccttg-3') and pBAD-sfGFP-F8TAG-R (5'-ctaaagttcttcaccttagaacctggttaattcc-3') were used for the construction of pBAD-sfGFP8TAG. Primers pBAD-sfGFP-F27TAG-F (5'-tctgtctgtggtgaaggtgaaggtgatg-3') and pBAD-sfGFP-F27TAG-R (5'-ctattatgaccattaacatcaccatcaagttc-3') were used for the construction of pBAD-sfGFP27TAG. Primers pBAD-sfGFP-F130TAG-F (5'-aaagaagagtaattcttctgtgcataaacttg-3') and pBAD-sfGFP-F130TAG-R (5'-ctaatcaatccttaagttcaatcagattaac-3') were used for the construction of pBAD-sfGFP130TAG.

Protein Expression and Purification. To express sfGFP incorporated with a NAA at its S2 position, *E. coli* BL21(DE3) cells were cotransformed with pEVOL-pylT-N346A/C348A and pET-sfGFP2TAG. Cells were recovered in 1 mL of LB media for 1 h at 37 °C and then plated on a LB agar plate that contained 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. A single colony was then selected and grown overnight in 10 mL of LB media. This overnight culture was used to inoculate 500 mL of GMML media supplemented with 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. Cells were grown at 37 °C in an incubating shaker (225 rpm), and protein expression was induced by the addition of 1 mM IPTG, 0.2% arabinose, and 2 mM of a NAA (for **19–21**, 4 mM was used) when OD₆₀₀ reached 0.5. After 12 h induction, cells were harvested, resuspended in a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and sonicated. The cell lysate was clarified by centrifugation (60 min, 11 000 rpm, 4 °C). The decanted clear supernatant was incubated with 3 mL of Ni²⁺-NTA resin (Qiagen) (2 h, 4 °C) and washed with 30 mL of the lysis buffer. Protein was finally eluted out using the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Eluted fractions were collected and concentrated. The collected fractions were further purified on an anion exchange column. The buffer was later changed to 10 mM ammonium bicarbonate using an Amicon Ultra-15 Centrifugal Filter Devices (10 000 MWCO, Millipore). The purified proteins were analyzed by 12% SDS-PAGE.

To express sfGFP27-**15**, *E. coli* Top10 cells were cotransformed with pEVOL-pylT-N346A/C348A and pBAD-sfGFP27TAG. Cells were recovered in 1 mL LB media for 1 h at 37 °C and then plated on a LB agar plate that contained 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. A single colony was then selected and grown overnight in 10 mL LB media. This overnight culture was used to inoculate 500 mL of LB media supplemented with 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. Cells were grown at 37 °C in an incubating shaker (225 rpm), and the expression of sfGFP27-**15** was induced by the addition of 0.2% arabinose and 2 mM **15** when OD₆₀₀ reached 0.5. The expressed sfGFP27-**15** was purified according to the same procedures for other sfGFP variants described above. To express sfGFP8-**15**, sfGFP130-**15**, and sfGFP135-**15**, plasmids pBAD-sfGFP8TAG, pBAD-sfGFP130TAG, and pBAD-sfGFP135TAG were used separately to transform Top10 cells that contained pEVOL-pylT-N346A/C348A. The following expression and purification procedures of sfGFP8-**15**, sfGFP130-**15**, and sfGFP135-**15** were the same as those for sfGFP27-**15**.

To express ¹⁵N-labeled sfGFP27-**15**, the Top10 cells transformed with pEVOL-pylT-N346A/C348A and pBAD-sfGFP27TAG were cultured in LB media to OD₆₀₀ 0.7–0.8. The cultured cells were collected and then transferred to GMML media with ¹⁵N-labeled ammonium chloride as a sole nitrogen source (¹⁵N, 99%, Cambridge Isotope Laboratories Inc.). The expression of ¹⁵N-labeled sfGFP27-**15** was induced by the addition of 0.2% arabinose and 1 mM **15** for 6 h. ¹⁵N-labeled wild-type sfGFP was expressed similarly using Top10 cells transformed with pBAD-sfGFP. The expressed proteins were purified similarly as other sfGFP variants.

ESI-MS Analysis of sfGFP Variants. ESI-MS experiments were performed using an Applied Biosystems QSTAR Pulsar (Concord, ON, Canada) equipped with a nanoelectrospray ion source. The MS data were acquired in positive ion mode (500–2000 Da) using a spray voltage of +2000 V and flow rate of 700 nL/min. Data analysis and

protein signal extraction were performed using the BioAnalyst software (Applied Biosystems). A mass range of m/z 500–2000 was used for spectral deconvolution, and the output range was 20 000 to 30 000 Da using a resolution of 0.1 Da and S/N threshold of 20. The final determined molecular weights of sfGFP variants have errors of roughly ± 1 Da.

NMR Measurements. sfGFP27-15 samples for ^{19}F NMR unfolding studies were prepared at a concentration of 0.7 mM in a buffer that contained 12 mM phosphate (pH 7.0), 140 mM NaCl, and 3 mM KCl. In the titration experiment, protein solution was diluted with the same buffer with or without GndCl and subsequently reconcentrated using a centrifugal concentration device. The concentration of GndCl at each titration point was determined based on ^1H NMR. Chemical shifts were referenced against an external standard of trifluoroacetic acid at -75.2 ppm in D_2O , which was located in the inner part of an NMR tube with two concentric compartments. ^{19}F spectra were recorded at a temperature of 25°C on a 400 MHz spectrometer equipped with a Broadband Observe (BBO) probe (Bruker Biospin). The ^{15}N -labeled sfGFP27-15 protein was prepared in the same solution as the unlabeled protein. The HMQC spectrum of ^{15}N -labeled sfGFP27-15 was recorded at 35°C on a 500 MHz NMR spectrometer equipped with a cryoprobe. ^{19}F NMR folding studies of other sfGFP variants were carried out similarly.

Fluorescence Equilibrium Unfolding Analysis. Different sfGFP proteins in PBS buffer were incubated with GndCl (1.0 to 7.0 M) for 12–48 h in RT. Their fluorescent intensities at 510 nm were then measured using a FL600 Microplate Fluorescence Reader (excitation wavelength, 450 nm). Midpoint denaturant concentrations of GndCl were determined from fitting the normalized fluorescent intensity (F) data to the equation $F = a + b/(1 + (C/C_m)^h)$, where a , b , C_m , and h are adjustable constants, and C is the molarity of the GndCl.

Sonogashira Cross-Coupling. To label sfGFP-13, a stock solution of 10 mM **D1** was prepared in DMSO and a stock solution of 80 mM sodium ascorbate was prepared in deionized H_2O . To an ice-chilled 0.75 mL centrifuge tube were added sequentially an aliquot of sfGFP-13 (0.69 μL , 2.0 mg mL^{-1}), an aliquot of 10 mM $\text{Pd}(\text{OAc})_2\text{:L1}$ complex solution (0.69 μL , 6.9 nmol), and an aliquot of sodium ascorbate (0.69 μL , 55.2 nmol), and the mixture was stirred at 37°C for 1 h to obtain an activated reagent cocktail. Eight microliters of 10 mM **D1** was then added to this activated reagent cocktail. The final mixture was stirred for 2 h. To label sfGFP-20, a stock solution of 10 mM **D3** was prepared in DMSO. To an ice-chilled 0.75 mL centrifuge tube were added sequentially an aliquot of **D3** (0.69 μL , 6.9 nmol), an aliquot of 10 mM $\text{Pd}(\text{OAc})_2\text{:L1}$ complex solution (0.69 μL , 6.9 nmol), and an aliquot of sodium ascorbate (0.69 μL , 55.2 nmol), and the mixture was stirred at 37°C for 1 h to obtain an activated reagent cocktail. Separately, to a 0.75 mL centrifuge tube containing 50 μL of PBS buffer was added 10 mM **D3** (6.21 μL , 62.1 nmol) and 10 μL of sfGFP-20 solutions (2.0 mg mL^{-1} in $1\times$ PBS buffer, 0.69 nmol). This mixture of **D3** and sfGFP-20 was then added to the previously activated reagent cocktail and stirred at 37°C for 2 h. The same two reactions were also carried out with wild-type sfGFP. The finally fluorescein-labeled proteins were analyzed by SDS-PAGE (12%) for fluorescence image (BioRad ChemiDoc XRS+) and further Coomassie blue staining.

Oxime Formation. sfGFP-19 (100 μL , 2 mg mL^{-1} in PBS buffer) was dialyzed against a 10 mM sodium acetate buffer (pH 4) and concentrated to 100 μL . **D2** (4 μL , 20 mM in DMSO) was then added and incubated at 25°C overnight. The same reaction was carried out with wild-type sfGFP. The finally labeled proteins were analyzed by SDS-PAGE (12%) for fluorescence image and further Coomassie blue staining.

Azide-alkyne Cycloaddition. For the copper-catalyzed azide-alkyne cycloaddition, to sfGFP-20 (0.5 mg mL^{-1} in PBS buffer, 161 μL , 2.78 nmol) or sfGFP-21 (2.6 mg mL^{-1} in PBS buffer, 31 μL , 2.78 nmol) was added CuSO_4 (100 μM final concentration), NiCl_2 (1 mM), $\text{tris}[(1\text{-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine$ (TBTA, stock solution in DMSO, 500 μM), and a corresponding dye **D4** or **D1** (in DMSO, 50 equiv of the protein) sequentially, followed by the addition of sodium ascorbate (5 mM). The reaction was performed at 25°C for

3 h. EDTA (0.5 M) was then added to the reaction mixture to chelate the two metals. The same reaction was carried out with wild-type sfGFP. The copper free cycloaddition reaction between sfGFP-21 (0.5 mg mL^{-1} in PBS buffer, 161 μL , 2.78 nmol) and **D5** (in DMSO, 50 equiv of the protein) was performed at 25°C overnight. The same reactions were carried out with wild-type sfGFP. All finally labeled proteins were analyzed by SDS-PAGE (12%) for fluorescence image and further Coomassie blue staining.

■ ASSOCIATED CONTENT

§ Supporting Information

Synthesis of 19–21, all DNA and protein sequences, and additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published to the Web on November 19, 2012, with an error in Figure 4. This was corrected in the version published to the Web on January 24, 2013.