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Incorporation of Unnatural Amino Acids in Response to the AGG Codon

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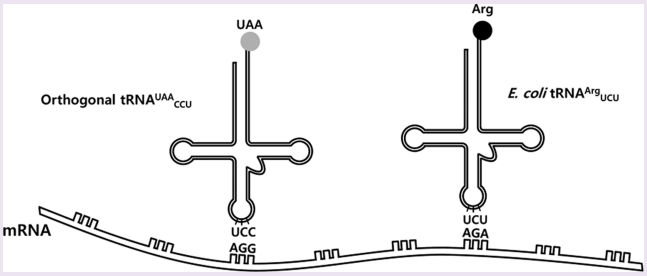
Supporting Information

ABSTRACT: The biological protein synthesis system has been engineered to incorporate unnatural amino acid into proteins, and this has opened up new routes for engineering proteins with novel compositions. While such systems have been successfully applied in research, there remains a need to develop new approaches with respect to the wider application of unnatural amino acids. In this study, we reported a strategy for incorporating unnatural amino acids into proteins by reassigning one of the Arg sense codons, the AGG codon. Using this method, several unnatural amino acids were quantitatively incorporated into the AGG site. Furthermore, we applied the method to multiple AGG sites, and even to tandem AGG sequences. The method developed and described here could be used for engineering proteins with diverse unnatural amino acids, particularly when employed in combination with other methods.



The addition of unnatural amino acids to the existing set of building blocks available for protein synthesis has provided researchers with an invaluable tool for manipulating protein structure and function and has thereby expanded the scope of protein engineering. Two methods have been developed independently and applied successfully to incorporate unnatural amino acids and engineer proteins with novel chemical moieties: site-specific incorporation into codons that are not generally used in organisms for coding amino acids (such as stop codons and four-base codons)[1−3] and residue-specific incorporation involving the reassignment of codons by replacement of one amino acid.[4−8] In particular, the site-specific incorporation system has been significantly advanced by the engineering of expression strains[9−12] and protein synthesis machinery.[13,14] Despite these advances, there remains a need to develop new strategies for incorporating unnatural amino acids into proteins, particularly in regard to wider applications (e.g., simultaneously, incorporation of diverse analogues into a protein), and one potential alternative approach is the reassignment of sense codons.[15,16]

Tirrell and co-workers previously reported a method for reassigning the UUU codon, one of two Phe codons (UUU and UUC), to L-3-(2-naphthyl)alanine (NaI) by introducing an engineered pair of phenylalanyl-tRNA synthetase and phenylalanyl-tRNA$_{AAA}$ (*yt*RNA$_{AAA}^{Phe}$) from *Saccharomyces cerevisiae*.[17] All the phenylalanyl-tRNAs of *Escherichia coli* have the same anticodon (GAA), and the tRNA reads the UUU codon through a G−U wobble base pair at the first position. In this scenario, the *yt*RNA$_{AAA}^{Phe}$ charged with NaI would interact with the UUU codon more strongly than the *E. coli* wild-type phenylananyl-tRNA$_{GAA}$ (tRNA$_{GAA}^{Phe}$) charged with Phe, in which the UUU codon might be reassigned to NaI. While the incorporation of NaI into the UUU codon was confirmed, the efficiency was measured around 80% based on mass spectrometry analysis of tryptic-digested peptides. The UUU codon was still read by the two tRNAs, *yt*RNA$_{AAA}^{phe}$ charged with NaI and tRNA$_{GAA}^{Phe}$ charged with Phe. We speculated that the abundance of tRNA$_{GAA}^{Phe}$ charged with Phe was the main reason why NaI was not quantitatively incorporated into the UUU site. Endogenous tRNA$_{GAA}^{Phe}$ charged with Phe is present at a high concentration to support the growth of the cell; thus, the preferential interaction of the Watson−Crick base pairing of A−U over the wobble base pairing of G-U might not be sufficient for a higher level of unnatural amino acid incorporation into the UUU codon.

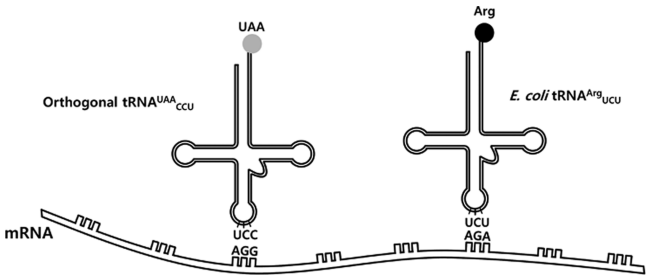
In order to overcome this particular limitation, we focused on different sense codons, rare codons that are defined as codons used rarely in genome. Another feature of rare codons is that they are usually decoded by low-abundant tRNAs.[18] Among the rare codons of *E. coli*, we chose to use the AGG codon, one of six codons for Arg. It is read by two tRNAs: the Watson−Crick base pairing with arginyl-tRNA$_{CCU}$ (tRNA$_{CCU}^{Arg}$) and a wobble

Received: January 21, 2015
Accepted: May 6, 2015
Published: May 6, 2015

DOI: 10.1021/acschembio.5b00230
ACS Chem. Biol. 2015, 10, 1648−1653

base paring of U−G with arginyl-$tRNA_{UCU}$ at the third position of the codon. Arginyl-$tRNA_{UCU}$ ($tRNA_{UCU}^{Arg}$) also recognizes the AGA codon, another rare codon. These facts suggest that the AGG codon could be decoded by $tRNA_{UCU}^{Arg}$ in the absence of $tRNA_{CCU}^{Arg}$, and under this condition, $tRNA_{CCU}$ charged with an unnatural amino acid can be used to incorporate the analogue into the AGG codon of recombinant proteins, similar to the UUU codon example given above. However, the important difference in our proposed method is that $tRNA_{UCU}^{Arg}$ is categorized as a low-abundance tRNA, which could improve the efficiency of unnatural amino acid incorporation into the sense codon of AGG. In addition, using this rare codon could reduce the impact of the unnatural amino acids on the proteome. In previous studies, reassignment of the AGG codon with unnatural amino acids was attempted via an *in vitro* protein translation system using the chemical misaminoacylation of $tRNA_{CCU}$[19] and an engineered pair of pyrrolysyl-tRNA synthetase and $tRNA^{Pyl}$.[20] Additionally, the possibility of reassigning the rare isoleucine AUA codon with unnatural amino acids was recently reported.[15] However, despite the attempts in reassigning sense codons with unnatural amino acids, quantitative incorporation has rarely been demonstrated. In the single successful example, researchers employed the incorporation machinery of selenocysteine; however, this system is currently limited to one amino acid analogue of selenocysteine.[21] Therefore, here we proposed a new strategy for the incorporation of unnatural amino acids into the rare Arg codon AGG (Scheme 1) and demonstrated the reassignment of the sense codon with several types of unnatural amino acids.

Scheme 1. Strategy for Incorporating Unnatural Amino Acids into the AGG Codon[a]

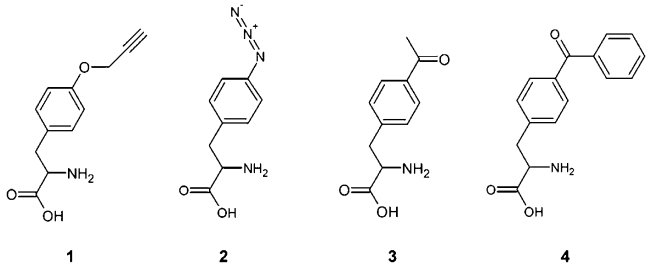


[a]In the proposed system, an orthogonal $tRNA_{CCU}$ charged with an unnatural amino acid is assigned into the AGG codon in the absence of endogenous $tRNA_{CCU}^{Arg}$, and the endogenous $tRNA_{UCU}^{Arg}$ is used for coding the AGA codon even though it naturally codes both the AGG and AGA codons. The base pairing between C and G has the thermodynamic advantage over the base pairing between U and G, and the concentration of $tRNA_{UCU}^{Arg}$ is low in *E. coli*.

First, we tested if $tRNA_{CCU}^{Arg}$ was necessary for the growth of *E. coli*. The gene for $tRNA_{CCU}^{Arg}$ (argW) was knocked out from the genome of *E. coli* DH10B using the homologous recombination method,[22,23] and the resulting strain was named *E. coli* BS01 (Figure S1a). Because it might need to control the concentration of Arg in the minimal medium, the gene encoding N-acetylglutamate synthase (argA), which is the first enzyme used in *E. coli* for the biosynthesis of Arg from Glu, was also knocked out (Figure S1b); however, we used a complex medium LB to incorporate the unnatural amino acids into the AGG codon (described below). The growth of the resulting knockout strain (*E. coli* BS02) in LB was compared with that of *E. coli* DH10B (Figure S2). The growth rates of the two strains were comparable, which indicated that the AGG codon could be read by $tRNA_{UCU}^{Arg}$ in the absence of $tRNA_{CCU}^{Arg}$, and that the concentration of $tRNA_{UCU}^{Arg}$ was sufficient to support the encoding of the AGG sites in the chromosome.

In our proposed method, an essential component is an orthogonal pair of $tRNA_{CCU}$ and an aminoacyl-tRNA synthetase charging the tRNA with unnatural amino acids (see Scheme 2). We focused on the orthogonal tyrosyl-

Scheme 2. Unnatural Amino Acids Used in This Study: 1, OpgY; 2, AzF, 3; AcF; 4, pBpa



$tRNA_{CUA}$ ($Mjt RNA_{CUA}$) and tyrosyl-tRNA synthetase pairs originating from *Methanococcus jannaschii*; these pairs have been engineered to incorporate a number of unnatural amino acids into the TAG stop codon.[1] The synthetase has shown a promiscuity toward the anticodon[24] even though its specificity can be improved;[13,25,26] therefore, we assumed that it might be active toward the $Mjt RNA_{CCU}$. The anticodon of an optimized $Mjt RNA_{CUA}$ was changed into CCU, and the tRNA gene was cloned into a plasmid (pEVOL[27]) encoding the engineered *M. jannaschii* tyrosyl-tRNA synthetase for *p*-azidophenylalanine (AzF-RS).[28] One unexpected property of AzF-RS is its ability to activate *O*-propargyl-tyrosine (OpgY), and this activity is higher than the *M. jannaschii* tyrosyl-tRNA synthetase originally engineered for OpgY (OpgY-RS;[29] Figure S3). Therefore, we decided to use AzF-RS for both AzF and OpgY. To examine the incorporation of unnatural amino acids into the AGG codon, we cloned the gene for the Z domain, including one AGG codon in its fifth position, into the pQE-80L plasmid. One additional plasmid was constructed for expression of *E. coli* prolyl-tRNA synthetase (ProRS) under the control of the tet promoter. It was recently reported that the *M. jannaschii* tyrosyl-tRNA synthetase, engineered for the incorporation of *p*-acetylphenylalanine (AcF) into the TAG codon, misaminoacylates the *E. coli* prolyl-tRNAs, and that this issue could be resolved by overexpressing the *E. coli* ProRS.[30] When examining the incorporation of OpgY into the proteome with AzF-RS and $Mjt RNA_{CUA}$ or $Mjt RNA_{CCU}$ via the Cu(I)-catalyzed click reaction[31] with biotin-azide, we observed high background signals and signals that were not distinct between the two *Mjt*RNAs (Figure S4). These results suggest that the background signals might not be related to the exogenously introduced tRNAs. Consistent with previous results for AcF-RS,[30] overexpressing the ProRS gene significantly reduced the background signal (Figure S4). We cotransformed BS02 with the plasmids for the $Mjt RNA_{CCU}$/AzF-RS pair and ProRS and investigated the incorporation of OpgY into the endogeneous proteins that naturally have the AGG codon. The unnatural amino acid was incorporated into the AGG positions of the *E. coli* proteome (Figure S5). We also compared the growth and viability of the strain with or without OpgY (Figure S6). It is

DOI: 10.1021/acschembio.5b00230
ACS Chem. Biol. 2015, 10, 1648−1653

not a surprise that the incorporation of OpgY into the endogeneous AGG codons hampered the growth of the strain and reduced the number of viable cells. Based on the results, we limited the expression time of recobminant proteins to 3 h, during which the OpgY incorporation into the *E. coli* proteome did not cause signficant differences in cell growth and viability.

Three plasmids, one for the *Mj*tRNA$_{CCU}$/AzF-RS pair, one for the Z domain, and one for ProRS, were transformed into *E. coli* DH10B or BS02, and the bacterial cells were grown in LB media. Expression of the two synthetases, AzF-RS and ProRS, was induced by adding 0.2% L-arabinose and 50 nM anhydrotetracycline, respectively, when OD$_{600}$ reached around 0.5. After 2 h, when OD$_{600}$ was around 1.0, the expression of the Z protein was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside in the presence of OpgY for 3 h. We used OpgY for initial experiments rather than AzF because of its higher efficiency click reaction for an azide group in cellular lysate compared to AzF for alkyne (data not shown). The cells were lysed with a 1% SDS solution, and the lysate was reacted with biotin-azide via the Cu(I)-catalyzed click reaction. The reaction products were analyzed by Western blot using streptavidin- or anti-His$_5$ antibody-horseradish peroxidase conjugates. The streptavidin signal was detected only when AzF-RS, *Mj*tRNA$_{CCU}$, and OpgY were all present and the reporter protein had the AGG codon (Figure 1a and Figure

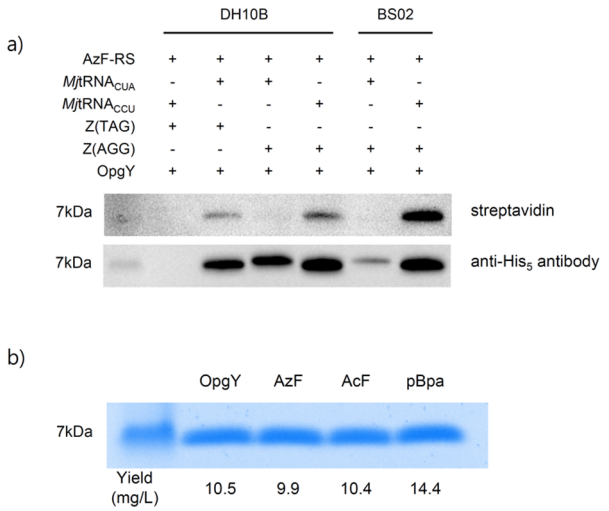


Figure 1. Incorporation of unnatural amino acids into the AGG codon. (a) Western blot analysis for the incorporation of OpgY in response to the AGG codon. Each cell lysate was subjected to a Cu(I)-catalyzed click reaction with biotin-azide, and then the blot was probed with streptavidin or anti-His$_5$ antibody. The sequences in parentheses indicate the codon in the fifth position of the Z domain. (b) SDS-PAGE analysis of purified Z domain expressed with various unnatural amino acids. Expression yields are included.

S7), which indicates that OpgY was incorporated into the AGG codon of the Z domain. The signal intensity for streptavidin relative to anti-His$_5$ antibody suggests that the knockout of argW improved the efficiency of OpgY incorporation in the AGG codon (lane 4 vs lane 6 in Figure 1a). In addition, the protein expression of Z(AGG) was compatible with that of the Z domain without the AGG codon (lane 1 vs lane 5 in Figure S7) and higher than that of Z(TAG) (lane 1 vs lane 6 in Figure 1a). To examine the efficiency of OpgY incorporation into the AGG codon, the purified Z(AGG) expressed in BS02 was analyzed using LC-MS. The observed masses were 8352.64 and 8366.63 Da, which correspond to Z-OpgY and most likely its methylated form, respectively (Figure 2a: the calculated mass of Z-OpgY is 8353.14 Da). An N-terminal methylation has previously been reported for proteins expressed in bacteria such as *E. coli*.[32−35] The mass peak for the Z domain that had Arg in the AGG codon (calculated mass: 8308.1 Da) was not detected in the mass spectrum. These results clearly indicate that the AGG codon was reassigned into OpgY in the presence of AzF-RS, *Mj*tRNA$_{CCU}$, and OpgY. The Z-OpgY protein was analyzed with LC-MS/MS after trypsin digestion, and the fragment including OpgY in the AGG position was detected (Table S1 and Figure S8). To acertain the requirement of ProRS overexpression, we expressed the Z(AGG) domain with OpgY in the absence of the plasmid for ProRS and analyzed the purified protein using LC-MS (Figure S9). Two additional masses were observsed compared to Z-OpgY with the ProRS plasmid (see Figure 2a), and they were matched with ones that OpgY is incoporated into one of the Pro positions of Z-OpgY and its methylated form.

Because the aminoacyl-tRNA synthetase used for OpgY was originally engineered for AzF, we expressed the Z domain with AzF instead of OpgY. In addition, to ascertain whether the method was applicable to other unnatural amino acids, we incorporated AcF and *p*-benzoylphenylalanine (pBpa) into the Z domain by using AcF-RS[36] and pBpa-RS,[37] respectively, which are engineered *M. jannaschii* tyrosyl-tRNA synthetases for the incorporation of their respective unnatural amino acids into the TAG codon. The expression level of the Z domains with the four unnatural amino acids was ∼10 mg/L. The masses of purified Z domains were analyzed using LC-MS. Identical to the results for OpgY, we detected two masses for each sample: one for the Z domain with unnatural amino acids and the other for its methylated form. For the AzF-incorporated sample, we observed two additional mass peaks that were smaller than Z-AzF and methylated Z-AzF by 26 Da. This was probably due to the decomposition of the azide group to amine.[38−40]

To test whether our method could be used for incorporation into multiple AGG codons, we constructed plasmids for GST-Z fusion proteins that had two (GST-Z-2AGG) or three AGG (GST-Z-3AGG) codons separated by the (GS) sequence (Figure 3a). We also constructed plasmids for the GST-Z fusion proteins with two (GST-Z-2tdAGG) or three tandem AGG codons (GST-Z-3tdAGG) to examine the effects of consecutive AGG sites. The fusion proteins were expressed with OpgY, and the incorporation of the alkyne group was analyzed by Western blot following a Cu(I)-catalyzed click reaction with a biotin-azide. The streptavidin signal increased as the number of AGG codons increased, but the signal for protein expression probed by anti-His$_5$ antibody was not dependent on the number of AGG sites (Figure 3b). The C-terminal fragments, including the linker and Z-domain, were purified by gel permeation chromatography after treatment with thrombin, and the masses of these fragments were analyzed by LC-MS (Figure S10 and Table S2). Even with three AGG codons, the incorporation of Arg into the AGG codon was rarely detected in the mass spectra. It should be noted that OpgY was efficiently incorporated into the three tandem AGG sites. In contrast to the expression of the Z domain, we did not observe the masses for methylated forms of the C-terminal Z domains, which lends support to a proposed explanation that N-terminal methylation resulted in masses that were 14 Da larger than the calculated masses observed in

DOI: 10.1021/acschembio.5b00230
ACS Chem. Biol. 2015, 10, 1648−1653

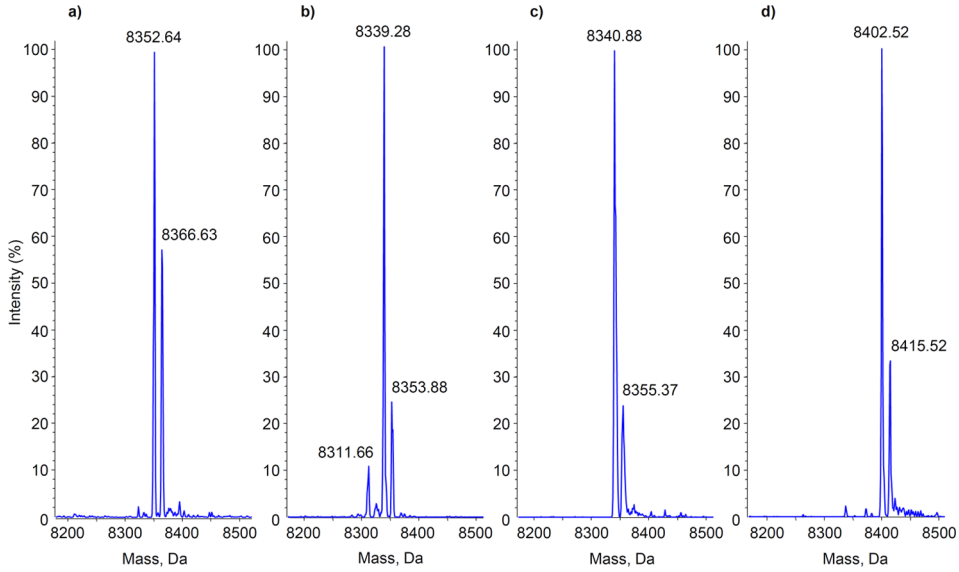


Figure 2. Deconvoluted ESI-mass spectra of the purified Z domains. (a) Z-OpgY (8353.14); (b) Z-AzF (8340.10); (c) Z-AcF (8341.12); (d) Z-pBpa (8403.20). The values in parentheses are the calculated masses of the Z domains when the proteins have an unnatural amino acid at the fifth position.

Figure 2. The incorporation of OpgY into the AGG codons was confirmed using LC-MS/MS for trypsin-digested GST-Z-3AGG (Figure 3c and Table S3). In addition, using the GST-Z fusion protein constructs, we tested the necessity of knocking out the argW gene from the *E. coli* chromosome for incorporating unnatural amino acids into the AGG codon. When expressed in *E. coli* DH10B, the masses for proteins in which Arg was incoporated into the AGG codon were detected (compare Figure S10 and S11). The results indicate that the removal of the competitive endogeneous $tRNA_{CCU}^{Arg}$ improved the incorporation efficiency of OpgY into the AGG codon to the quantitative level.

We have proposed and demonstrated a new strategy for the incorporation of unnatural amino acids into proteins. Our method involved reassignment of the AGG sense codon using an orthogonal pair of $tRNA_{CCU}$ and an aminoacyl-tRNA synthetase charging the tRNA with the unnatural amino acid. We succeeded in quantitatively incorporating several unnatural amino acids into the AGG sites of the protein. Furthermore, the method worked efficiently for multiple AGG sites, including a tandem sequence of three AGG codons. We expect that our method could be used in combination with existing methods to simultaneously incorporate diverse unnatural amino acids into proteins.

METHODS

Strain Construction. Knockout of argW or argA genes was performed according to the published method.[23] A kanamycin resistant cassette was amplified from a pKD13 plasmid using a set of primers including homologous regions to the target gene (primers 1 and 2 for argW; primers 3 and 4 for argA), and the PCR product was incubated with DpnI to remove the template plasmid. Subsequently, 400 ng of the purified PCR product was electroporated into DH10B containing a pKD46 plasmid that encodes λRed Recombinase genes, and the cells were recovered with SOC including 1 mM L-arabinose. After incubation at 37 °C for 2 h, the cells were spread onto LB agar plates including kanamycin and incubated at 37 °C. Using colony PCR, several colonies were tested for the location of the kanamycin resistance cassette in the chromosome (primers 5 and 6 for argW; primers 5 and 7 for argA). The kanamycin resistance cassette was removed from the chromosome via transformation with a pCP20 plasmid encoding FLP recombinase. The deletion of the kanamycin resistance cassette was also confirmed via colony PCR (primers 8 and 9 for argW; primers 10 and 7 for argA).

Plasmid Construction. The pEVOL plasmid[27] constructed by the Schultz group was used for expressing orthogonal aminoacyl-tRNA synthetase and tRNA pairs following a modification. The plasmid has two copies of aminoacyl-tRNA synthetase genes, one under the araBAD promoter and the other under the gln *S* promoter, and the latter expression cassette was not used in this study. We observed no difference in levels of aminoacyl-tRNA synthetase expression when using a high concentration of arabinose such as 0.2%. Thus, the codon-optimized synthetic genes of the aminoacyl-tRNA synthetase were cloned under the araBAD promoter using the *Bgl*II and *Sal*I sites. The anticodon (CUA) of tRNA in pEVOL was changed into CCU by site-directed mutagenesis (using primers 11 and 12). We constructed two types of plasmid for expressing model proteins in this study. For the first plasmid, the codon-optimized synthetic gene of the Z domain was cloned in the pQE-80L plasmid using the *Bam*HI and *Hin*dIII sites, and then the AGG or TAG site was introduced into the fifth position of the Z domain using site-directed mutagenesis (primers 13 and 14 for AGG; primers 15 and 16 for TAG). The original amino acid was Phe. The second type of plasmid was used to examine multiple-site incorporation of unnatural amino acids. It was constructed by cloning the Z domain gene into pGEX 4T-1 using the *Eco*RI and XhoI sites (primer 17 and primer 18). Linker sequences, including one to three AGG codons with either a Gly-Ser sequence space (primers 21 and 22 for GST-Z-1AGG, primers 23 and 24 for GST-Z-2AGG, primers 35 and 26 for GST-Z-3AGG) or in tandem (primers 27 and 28 for GST-Z-2tdAGG, primers 29 and 30 for GST-Z-3tdAGG), were annealed, phosphorylated using T4 polynucleotide kinase (3′ phosphatase minus), and then cloned using the *Bam*HI and *Eco*RI sites. The proS gene for prolyl-tRNA synthetase (ProRS) was amplified from the chromosome of *E. coli* DH10B using PCR (primer 19 and 20; primer 19 includes a ribosome binding site) and then cloned under the tetracycline inducible promoter of pBbs2K[41] using the *Bgl*II and *Bam*HI sites.

Incorporation of Unnatural Amino Acids into Protein. Three plasmids, one for expression of the orthogonal pairs of aminoacyl-tRNA synthetase and tRNA, one for the model proteins of the Z domain or GST-Z domain, and one for ProRS, were transformed into *E. coli* DH10B or BS02 strains. The cells were cultured in LB at 37 °C until OD_{600} reached ∼0.5, and then 0.2% L-arabinose and 50 nM

DOI: 10.1021/acschembio.5b00230
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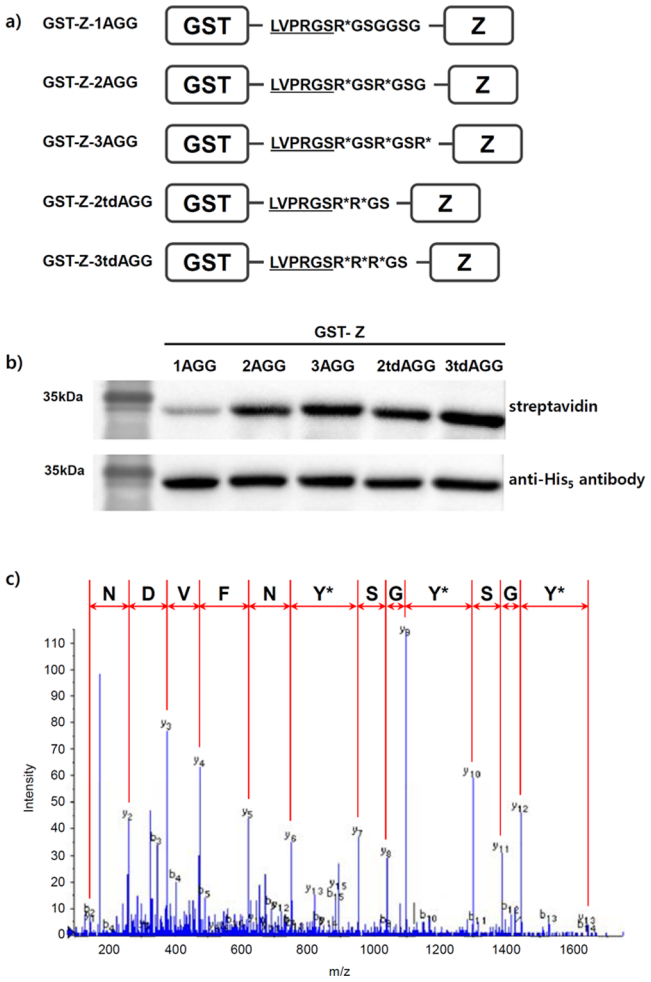

Figure 3. Multiple-site incorporation of OpgY via the AGG codon. (a) Schematic structures of the GST-linker-Z fusion proteins. The underlined sequence of LVPRGS is the thrombin cleavage site, and R* indicates the AGG codon site. (b) Western blot analysis for incorporation of OpgY into multiple AGG codons. Cell lysates expressing each construct were reacted with biotin-azide via the Cu(I)-catalyzed click reaction, and then the blot was probed with streptavidin or an anti-His_5 antibody. (c) MS/MS spectrum of the linker peptide (GSY*GSY*GSY*EFVDNK; Y*: OpgY) from GST-Z-3AGG. The y ions for the partial sequence Y*GSY*GSY*EFVDN are indicated by red lines. The sequences and masses of the b and y fragments are shown in Table S2.

anhydrotetracycline were added to the culture to induce expression of the orthogonal aminoacyl-tRNA synthetase and ProRS. When OD_{600} reached 1.0, 1 mM isopropyl β-D-1-thiogalactopyranoside and 2 mM of the unnatural amino acids were added to the culture to induce expression of the model proteins. After 3 h, the cells were harvested by centrifugation (9300*g* at 4 °C for 15 min), and the cell pellets were stored at −20 °C until they were used.

Copper-catalyzed Click Reaction and Western Blot Analysis. Biotin-PEG3-azide (Click Chemistry Tools) was conjugated to the alkyne group in proteins by using the Cu(I)-catalyzed click reaction. Cells were resuspended in a 1% SDS solution and then heated at 90 °C for 5 min. The cellular lysate was centrifuged at 18 000*g* for 5 min. The supernatant was used for analysis via a click reaction, conducted at RT for 1 h in the following solution: 400 μM biotin-PEG_3-azide, 0.5 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 0.1 mM $CuSO_4$, 5 mM aminoguanidine, and 5 mM sodium ascorbate in 100 mM phosphate buffer (pH 7.0).[31] The reaction product was analyzed by a Western blot using anti-His_5 antibody HRP conjugate (Sigma-Aldrich) or streptavidin HRP conjugate (Click Chemistry Tools), and the signal was obtained using the ChemiDoc XRS system (Bio-Rad).

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental results and methods. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00230.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. P.G. Schultz for the pEVOL plasmids. This research was supported by the Basic Science Research Programs (2012R1A2A2A02016803, 2012R1A1A10017878, and NRF-2009-0093826) and the Pioneer Research Center Program (2014M3C1A3051460) through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning, and the Ministry of Education. This research was also partially supported by the Ajou University research fund.

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