

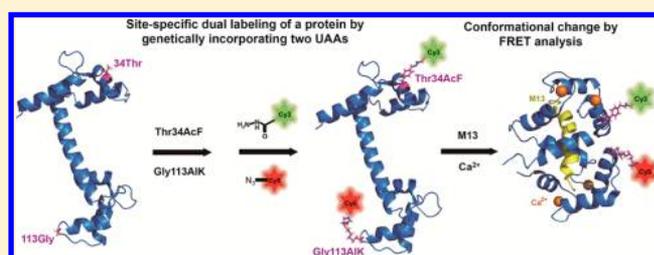
Simple and Efficient Strategy for Site-Specific Dual Labeling of Proteins for Single-Molecule Fluorescence Resonance Energy Transfer Analysis

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S Supporting Information

ABSTRACT: Analysis of protein dynamics using single-molecule fluorescence resonance energy transfer (smFRET) is widely used to understand the structure and function of proteins. Nonetheless, site-specific labeling of proteins with a pair of donor and acceptor dyes still remains a challenge. Here we present a general and facile method for site-specific dual labeling of proteins by incorporating two different, readily available, unnatural amino acids (*p*-acetylphenylalanine and alkylnyllysine) for smFRET. We used newly evolved alkylnyllysine-specific aminoacyl-tRNA synthetase/tRNA_{UCA} and *p*-acetylphenylalanyl-tRNA synthetase/tRNA_{CUA}. The utility of our approach was demonstrated by analyzing the conformational change of dual-labeled calmodulin using smFRET measurements. The present labeling approach is devoid of major limitations in conventional cysteine-based labeling. Therefore, our method will significantly increase the repertoire of proteins available for FRET study and expand our ability to explore more complicated molecular dynamics.



Analyzing the conformational dynamics of biological macromolecules including proteins is essential to a better understanding of complex biological systems. Single-molecule fluorescence resonance energy transfer (smFRET) has proved to be a powerful means to explore the dynamics of biological macromolecules at the single-molecule level. Since smFRET measures the changes in the distance between the donor and acceptor fluorophores, labeling of biological macromolecules with two different dyes at defined positions is crucial. Nucleic acids are relatively easy and simple to label with dyes, so studies on nucleic acid-interacting proteins using smFRET have flourished in recent years.^{1–3}

Despite many advances, however, smFRET analysis of proteins in general is quite limited, largely because labeling with multiple fluorophores at specific positions is still challenging. For site-specific tagging, the cysteine–maleimide method has been widely used. This approach, however, critically suffers from a lack of general applicability. It brings about multiple point mutations both to remove unwanted cysteine residues and to introduce two cysteine residues at desired labeling sites, which often leads to loss of biological activity of target proteins. In addition, random labeling of cysteine residues gives rise to heterogeneously labeled populations, which causes serious problems for precise FRET analysis and makes it almost impossible to achieve selective multiple (e.g., triple) labeling. Position-specific incorporation of two different fluorescent amino acids using a cell-free

translation system was reported,⁴ but multistep chemical synthesis is a discouraging bottleneck.

In an attempt to expand the chemical and biological functionalities of proteins, genetic incorporation of an unnatural amino acid (UAA) into a protein was developed.^{5–7}

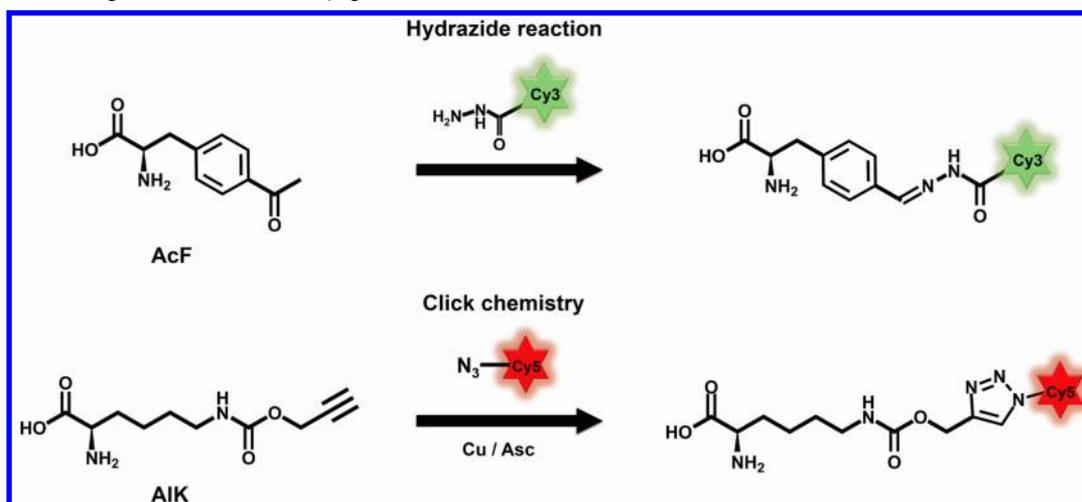
We and others demonstrated that site-specific protein labeling through incorporation of a UAA and subsequent conjugation with a fluorophore by bio-orthogonal chemistry are useful for efficient FRET analysis.^{8–10} These approaches, however, allow single bio-orthogonal conjugation, thus generating a single labeling. Additional introduction of a cysteine residue into a protein is still generally used for second color labeling via cysteine–maleimide chemistry. Hence, smFRET analysis is mostly limited to a subset of proteins that are tolerable to cysteine mutations, which constitute only a small fraction of proteins of interest in the biological system. Genetic incorporation of different UAAs was previously reported.^{11,12} This approach in theory can lead to protein dual labeling through distinct bio-orthogonal conjugations. However, the incorporated UAAs were azido and alkylnyl amino acids, which allowed only single bio-orthogonal conjugation through click chemistry.¹² Very recently, dual labeling of proteins by incorporating two UAAs carrying different bio-orthogonal

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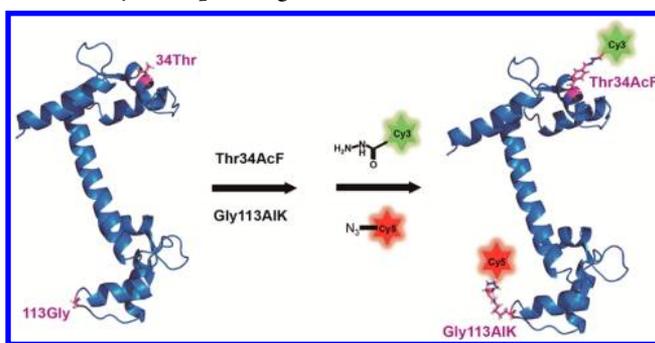
Scheme 1. Bio-Orthogonal Chemical Conjugation Reactions between Unnatural Amino Acids and Related Fluorescent Dyes



reactive groups was reported.¹³ This elegant approach, however, requires multistep organic synthesis for the preparation of both UAA and dyes, which can limit its application.

Here we present a general approach to site-specific dual labeling of a protein by genetically incorporating two different, readily available UAAs. To this end, we first chose two UAAs, alkynyllysine and *p*-acetylphenylalanine, because they are easy to obtain and contain distinct bio-orthogonal functionality for labeling (Scheme 1). For efficient genetic incorporation of alkynyllysine, we designed and evolved alkynyllysine-specific aminoacyl-tRNA synthetase. With the newly developed alkynyllysyl-tRNA synthetase (AIKRS)/tRNA_{UCA} and previously reported *p*-acetylphenylalanyl-tRNA synthetase (AcFRS)/tRNA_{CUA} pairs, we simultaneously incorporated the two different unnatural amino acids into specific positions of calmodulin (Scheme 2). The protein was then labeled with

Scheme 2. Site-Specific Dual Labeling of a Protein by Genetically Incorporating Two UAAs



the donor (Cy3; Cy = cyanine) and acceptor (Cy5) dyes, and a conformational change of calmodulin was investigated by ensemble and single-molecule FRET analysis.

EXPERIMENTAL SECTION

Materials. Cy3-hydrazide was purchased from GE Healthcare and Cy5-azide from Lumiprobe. M13 skeletal muscle myosin light-chain kinase peptide (SK-MLCK M13) was purchased from ANASPEC and 4-hydroxyazobenzene-2-carboxylic acid (HABA)/avidin from Sigma. *p*-Acetylphenyla-

lanine was obtained from Chem-Impex, and alkynyllysine was simply synthesized in two steps.⁹ Expression vectors pCDFDuet, pRSFDuet, and pACYCDuet were purchased from Novagen.

Evolution of AIKRS. For increased expression of *Methanosarcina mazei* PylRS (MmPylRS; PylRS = pyrrolysyl-tRNA synthetase), the nucleotide sequence was codon-optimized for *Escherichia coli* (the nucleotide sequence of codon-optimized MmPylRS is listed below). The resulting MmPylRS gene was cloned into *SacI* and *AscI* of the pKD library vector,¹⁴ and as a result five amino acids (Met-Glu-Phe-Glu-Leu) were additionally attached to the N-terminus. To evolve MmPylRS for the incorporation of alkynyllysine (AIK), five residues (L301, L305, Y306, L309, and C348) in the active site of MmPylRS were randomly mutated by using the primers MMO301569X (CTTCTGCCTGCGTCCGATGNNKGCACCGAACNNKNNKAACTACNNKCGTAAGCTGGATCGCGCC), MMO300r (CGGACGCAGGCAGAAGTT), MM0348X (GTTCCACCATGCTGAACTTCNNKCGATGGGCAGCGGTTGC), and MMO348r (GAAGTTCAGCATGGTGAAC). Amplified polymerase chain reaction (PCR) products were introduced into the pKD library vector,¹⁴ resulting in a pKD-MmPylRS library. The active synthetase variants were selected through several positive and negative selections. In positive selection, the pKD-MmPylRS library plasmid was transformed to *E. coli* TOP10 competent cells which contain the pCAT-pylT plasmid carrying tRNA^{Pyl} and the chloramphenicol acetyl transferase (CAT) gene with a TAG stop codon at Asp-112. The transformants were recovered in 40 mL of SOC at 30 °C for 2 h and plated on lysogeny broth (LB) plates containing 10 μg/mL tetracycline (TC), 50 μg/mL kanamycin (Km), 40 μg/mL chloramphenicol (Cm), 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and 0.2 mM AIK. After incubation at 30 °C for 36 h, all colonies were collected. For negative selection, the library plasmids were extracted from the selected cells and introduced into chemically competent *E. coli* TOP10 with the negative selection plasmid pAraCB2-pylT.¹⁵ After the cell was recovered in 40 mL of super optimal broth with catabolite repression (SOC) at 30 °C for 2 h, transformants were spread on the LB plate (containing 30 μg/mL Cm, 50 μg/mL Km, and 0.1 mM IPTG) in the absence of the AIK at 30 °C for 36 h. The surviving colonies were collected, and pKD-MmPylRS library plasmids were

isolated for additional positive selection. Twenty-three colonies were selected from the agar plate containing 40 $\mu\text{g}/\text{mL}$ Cm, and an individual clone was checked for AIK activity using 10, 20, 40, 60, and 80 $\mu\text{g}/\text{mL}$ Cm agar plates. After 36 h at 30 °C, five clones were found to have an IC_{50} value of 60 $\mu\text{g}/\text{mL}$ Cm in the presence of AIK but 2.5 $\mu\text{g}/\text{mL}$ Cm in the absence of AIK.

Calmodulin Expression and Purification. To express calmodulin (CaM) mutants with unnatural amino acids, the plasmid pCDFDuet-BAP-CaM(34TAG, 113TGA) was cotransformed with pRSFDuet-AcFRS-tRNA_{CUA} and pACYCDuet-AIKRS-tRNA_{UCA} into BL21(DE3). As a control, wild-type CaM was expressed. Cultures were grown in 200 mL of terrific broth (TB) medium containing antibiotics at 37 °C. When OD_{600} reached 0.6, the cells were induced with 0.5 mM IPTG, 1 mM *p*-acetylphenylalanine (AcF), and 1 mM AIK. After 12 h at 30 °C, the cells were harvested and sonicated in 10 mL of lysis buffer (50 mM Tris-HCl (pH 7.8), 300 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM imidazole, 0.1 mg/mL lysozyme, and protease inhibitor cocktail (Roche)). The lysate was centrifuged at 15 000 rpm at 4 °C for 15 min, and clarified supernatant was applied to Ni-NTA (NTA = nitrilotriacetic acid) agarose (Qiagen). CaM was washed with wash buffer (50 mM Tris-HCl (pH 7.8), 300 mM NaCl, 10 mM 2-mercaptoethanol, 40 mM imidazole) and eluted using elution buffer (50 mM Tris-HCl (pH 7.8), 300 mM NaCl, 10 mM 2-mercaptoethanol, 250 mM imidazole). CaM was further purified by using an anion exchange column (HiTrap Q HP, GE Healthcare) connected to a fast protein liquid chromatography (FPLC) system (AKTAprime plus, GE Healthcare).

In Vitro Biotinylation. For in vitro biotinylation of CaM, biotin-protein ligase (BirA enzyme) containing a C-terminal hexahistidine tag was purified. CaM was biotinylated with purified BirA enzyme in 50 mM bicine buffer with 10 mM magnesium acetate, 10 mM ATP, and 50 μM biotin at 30 °C for 12 h.

Confirmation of Biotinylation (Western Blot and HABA/Avidin). Biotinylated CaM was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, BIO-RAD) in transfer buffer (25 mM Tris base, 19 mM glycine, 20% methanol). After being washed with phosphate-buffered saline (PBS), the membrane was blocked using TPBSA buffer (PBS + 0.5% bovine serum albumin (BSA) + 0.05% Tween-20) for 1 h at room temperature. Streptavidin-horseradish peroxidase (HRP) (R&D Systems) was added to the membrane, and the mixture was incubated for 1 h. Biotinylated CaM was detected by 4-chloro-1-naphthol (4-CN).

Labeling of CaM Mutants. Cy5-azide dye was first conjugated with the alkyne group of AIK-containing CaM using click chemistry as described before^{10,16} with minor modification. Briefly, a 20-fold excess of Cy5-azide was mixed with CaM in the presence of 1 mM CuSO_4 and 1 mM sodium ascorbate. The mixture was incubated in the dark at 4 °C for 12 h. Then Cy3-hydrazide was added to the reaction mixture. After gentle agitation of the mixture at ambient temperature for 3 h, labeled CaM was eluted using a PD-10 desalting column with 25 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl for removal of unreacted free dye. The dye to protein ratio (Cy3/CaM or Cy5/CaM) was determined by measuring the absorbance at 280, 552, and 650 nm for CaM, Cy3, and Cy5 respectively. Extinction coefficients of CaM ($7000 \text{ M}^{-1} \text{ cm}^{-1}$ at

280 nm), Cy3 ($150\,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 552 nm), and Cy5 ($250\,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 650 nm) were used to calculate the molar concentration of the protein and the dyes.

Fluorescence Scanning (Transfer and Scan). Dual-labeled CaM was loaded onto the 15% SDS-PAGE system and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, BIO-RAD). The membrane was scanned for Cy3 and Cy5 using a scanner (GenePix Personal 4100A, HP).

Ensemble FRET Experiments. A 0.5 μM concentration of labeled CaM was mixed with 25 mM Tris-HCl buffer (pH 7.5, 100 mM KCl) containing 1 mM CaCl_2 and 10 μM M13 peptide (in the presence or absence of 10 mM ethylene glycol tetraacetic acid (EGTA)) in a 96-well plate (Nunc), and the mixture was incubated in the dark for 10 min at room temperature. Emission spectra of CaM were read in 2 nm increments from 562 to 700 nm using a plate reader (Infinite M200, TECAN) under 532 nm excitation.

Single-Molecule FRET Experiments. Single-molecule FRET analysis was conducted as previously described.¹⁰ Biotinylated and dual-labeled CaM was diluted to a final concentration of 0.5 nM and incubated with 1 mM CaCl_2 in the presence or absence of 10 μM M13 peptide. The resulting CaM was injected into a sample chamber and immobilized on the PEG-coated surface via biotin-neutravidin interaction through incubation for 5 min. The channel was washed with 25 mM Tris-HCl buffer (pH 7.5, 100 mM KCl, 1 mM CaCl_2) with or without M13 peptide and EGTA to remove free proteins. We collected the molecules that clearly showed a single bleaching step for a donor and an acceptor, indicating a single pair of the donor and acceptor dyes. Hundreds of molecules for each condition were collected. The FRET efficiency of individual proteins was determined on the basis of the most commonly used intensity-based equation, $I_A/(I_D + I_A)$, as described elsewhere.¹⁷ I_D and I_A are the fluorescence intensities of the donor (Cy3) and acceptor (Cy5) of each single molecule, respectively. The acceptor emission signal can be affected by the contribution from donor emission and direct excitation of the acceptor; in the case of Cy3-Cy5, the former usually lies in the range of 8–15% of the donor emission and the latter is known to be negligible.¹⁸ Thus, to minimize the background noise and the contribution effect of the donor emission, the following procedure was employed. The average fluorescence intensities from the donor and acceptor channels were measured before immobilization of the sample, and their intensities were considered as the background fluorescence and subtracted uniformly from the fluorescence intensities from individual proteins. The leakage of donor fluorescence into the acceptor channel and that of acceptor fluorescence into the donor channel were also taken into account as described elsewhere.^{10,18} The FRET efficiency histogram of CaM was fitted by the Gaussian function using OriginPro 8 (OriginLab Corp.).

Mass Spectrometry. Purified protein was loaded onto the 15% SDS-PAGE system, and then each protein band was excised from the gel. The sliced gel was washed five times with 150 μL of 1:1 acetonitrile/25 mM ammonium bicarbonate (pH 7.8). The gel slices were dried in a Speedvac concentrator and then rehydrated with 30 μL of 25 mM ammonium bicarbonate (pH 7.8) containing 20 ng of trypsin (Promega, Madison, WI) for 20 h at 37 °C. To extract the remaining tryptic peptides, the gel was incubated with 20 μL of 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid for 40 min at 30 °C. The combined supernatants were evaporated in a Speedvac

concentrator and dissolved in 8 μL of 5% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) formic acid for mass spectrometric analysis. The tryptic peptides were analyzed using reversed-phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer. A sample was bound to the trapping column (0.1 \times 20 mm trapping and 0.075 \times 130 mm resolving columns) packed with Vydac 218MS low trifluoroacetic acid C18 beads (5 μm , 300 \AA pore size; Vydac, Hesperia, CA) for 10 min with 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. Peptide fragments were eluted with a 50 min gradient of 5–80% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.2 $\mu\text{L}/\text{min}$. For tandem mass spectrometry, a full mass scan range mode was $m/z = 450\text{--}2000$ Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with a relative collision energy of 55%. The individual spectra from MS/MS were processed using the TurboSEQUENT software (Thermo Quest, San Jose, CA). The generated peak list files were used to query either the Mass Spectrometry Protein Sequence Database (MSDB) or National Center for Biotechnology Information (NCBI) using the MASCOT program (<http://www.matrixscience.com>). Modifications of methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by MASCOT probability analysis were considered initially.

RESULTS AND DISCUSSION

Molecular Evolution of Alkynyllysine-Specific Aminoacyl-tRNA Synthetase. To develop a more versatile approach to site-specific dual labeling of proteins, we attempted genetic incorporation of two different UAAs enabling distinct bio-orthogonal conjugations. We selected previously engineered AcFRS/tRNA_{CUA} and natural PylRS/tRNA_{CUA} pairs to incorporate AcF and AIK for ketone–oxamine and click reactions, respectively.^{8,9} We chose these two UAAs because they are known to allow mild, bio-orthogonal, and highly efficient conjugations (Scheme 1).^{8,9} In addition, both UAAs are readily available, AcF from a commercial source and AIK by a simple and high-yield synthesis.⁹

We first synthesized *E. coli* codon-optimized genes of AcFRS and PylRS derived from *Methanococcus jannaschii* and *Methanosarcina barkeri*, respectively (see the Supporting Information). We then examined the incorporation capability of the selected aminoacyl-tRNA synthetase/tRNA pairs by measuring the read-through efficiency of the CAT gene carrying an amber codon at position 112. In vivo assay with CAT or LacZ has been used as a standard measure of the suppression efficiency of a natural or evolved orthogonal aminoacyl-tRNA synthetase.^{14,15} The half-maximal inhibitory concentration (IC₅₀) values are indicative of the amount of the CAT enzyme produced by amber codon suppression. When AcFRS/tRNA_{CUA} was expressed, Cm resistance was increased from 2.5 to 15 $\mu\text{g}/\text{mL}$ by the presence of AcF (Figure 1a). The MbPylRS/tRNA_{CUA} pair, however, showed only a subtle change in IC₅₀ values (from 2.5 to 5 $\mu\text{g}/\text{mL}$) with additional AIK (Figure 1a). MbPylRS was previously shown to be capable of incorporating AIK,⁹ but unfortunately the CAT-based suppression was not substantial under our experimental conditions (Figure 1b).

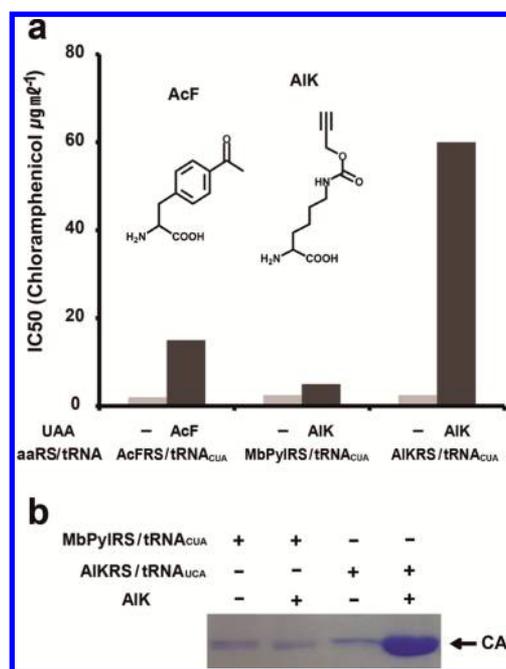


Figure 1. Molecular evolution of AIKRS for incorporation of AIK. (a) In vivo CAT assay using various aminoacyl-tRNA synthetase/tRNA pairs. (b) Production of full-length CAT by MbPylRS/tRNA_{CUA} and AIKRS/tRNA_{CUA}.

The yield of recombinant proteins containing a UAA is critically dependent upon the in vivo aminoacylation activity of an orthogonal aminoacyl-tRNA synthetase.^{14,15} Thus, we attempted molecular evolution of MmPylRS for improved incorporation of AIK. A library of MmPylRS variants (1.0×10^7) was constructed by introducing random mutations at five residues (L301, L305, Y306, L309, and C348) that constitute the amino acid binding pocket of the synthetase.^{19,20} The resulting library was introduced into *E. coli* TOP10 carrying tRNA^{Pyl} and subjected to a series of alternating CAT- and control of cell death protein (CcdB)-based selections as described.^{14,15} Twenty-three clones were picked after a second round of positive CAT selection, and of them five clones showed AIK-dependent growth on the LB–Cm agar plate. Sequence analysis revealed that they have the same mutations; L301M and Y306L. In the subsequent in vivo CAT assay, the finally selected synthetase displayed high suppression activity (IC₅₀ = 60 $\mu\text{g}/\text{mL}$) for AIK while maintaining low background activity in the absence of AIK (Figure 1a). SDS–PAGE analysis of the suppressed CAT protein confirmed high in vivo aminoacylation activity of the evolved synthetase (Figure 1b). These results illustrate that AIK-specific synthetase (designated alkynyllysyl-tRNA synthetase) was successfully evolved.

Production of Calmodulin Carrying *p*-Acetylphenylalanine and Alkynyllysine at Specific Positions. To examine the general applicability of a simultaneous AcF and AIK incorporation system to site-specific dual labeling of a protein for smFRET analysis, we chose CaM as a model protein, since it is known to undergo notable conformational changes when it binds to Ca²⁺ and the M13 peptide.²¹ CaM has four EF-hand calcium binding motifs, EF1 and EF2 in the N-terminal domain and EF3 and EF4 in the C-terminal domain. Since the distance between the two domains changes markedly on binding of target peptides, we picked several residues in EF1 (Thr34 and Ser38) and EF3 (Thr110, Gly113, and Lys115)

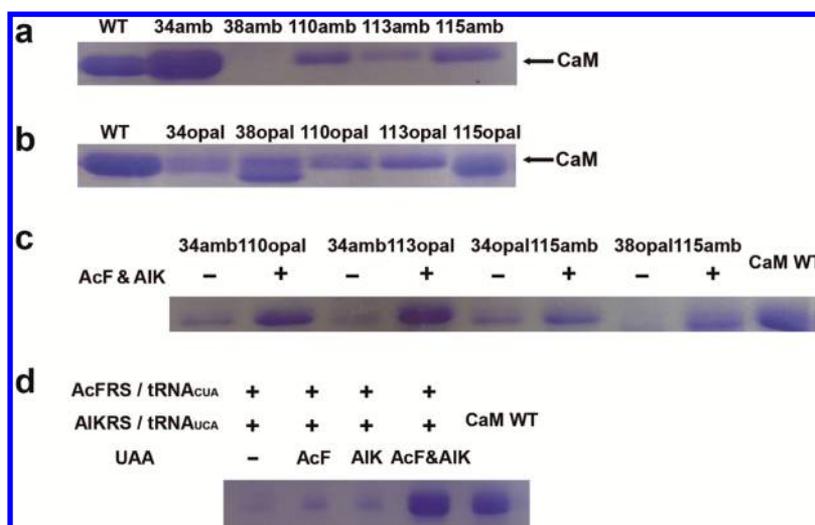


Figure 2. Expression of full-length CaM variants. (a) Suppression of amber stop codons of CaM by AcF and AcFRS/tRNA_{CUA}. (b) Suppression of opal stop codons of CaM by AIK and AIKRS/tRNA_{UCA}. (c) Production of CaM with double stop codons by AcFRS/tRNA_{CUA} and AIKRS/tRNA_{UCA} in the presence or absence of UAAs.

and tested the UAA incorporation efficiency of each site using amber and opal codons. Previous studies revealed that amber, opal, ochre, and quadruplet codons are useful for single and double incorporation of UAAs.^{11,12} *E. coli* BL21 carrying CaM with a C-terminal hexahistidine tag and amber or opal codons at various positions was grown in the presence of UAAs (1 mM). AcFRS/tRNA_{CUA} or AIKRS/tRNA_{UCA} (an opal suppressor tRNA of pylT) was additionally added to the host strain to evaluate the suppression efficiency of the amber or opal codon, respectively. In the amber suppression test, CaM-34amb, CaM-110amb, and CaM-115amb exhibited better suppression by AcFRS/tRNA_{CUA} and AcF than CaM-38amb and CaM-113amb (Figure 2a). The opal suppression analysis revealed that all the tested CaM variants showed good suppression by AIKRS/tRNA_{UCA} and AIK (Figure 2b).

On the basis of the above results, we constructed four different CaM variants containing amber and opal codons at designated sites (34amb110opal, 34amb113opal, 34opal115amb, and 38opal115amb). Then we tested the production of full-length CaM suppressed by both AcFRS/tRNA_{CUA} and AIKRS/tRNA_{UCA} pairs in the presence or absence of UAAs. All the constructs tested exhibited good suppression by UAAs and low background (Figure 2c). Since CaM-34amb113opal displayed the highest suppression and lowest background, this construct was selected for further analysis. Significant amounts of full-length CaM (3 mg/L culture) were produced when both AcF and AIK were supplemented in the medium, whereas the absence of either UAA only resulted in unproductive CaM expression (Figure 2d). When a natural MbPylRS instead of an engineered AIKRS was used to produce CaM using the same construct, CaM-34amb113opal, only trivial amounts of full-length CaM were obtained (Figure S-1 in the Supporting Information). LC-MS/MS analysis of the CaM variant carrying AcF and AIK (CaM-AcF-AIK) verified the incorporation of the two UAAs at the designated positions (Figure S-2 in the Supporting Information).

Dual Labeling of Calmodulin. For dual labeling of CaM-AcF-AIK, commercial dyes with bio-orthogonal functional groups (Cy3-hydrazide and Cy5-azide) were used (Scheme 1). CaM-AcF-AIK was first conjugated with an acceptor dye, Cy5-azide, through click reaction between the alkyne and azide

groups in the presence of 1 mM CuSO₄ and sodium ascorbate in bis-Tris buffer (pH 7.0) as described before.^{10,16} Unlike the previous report,¹³ protein oxidation or aggregation was not observed during copper-catalyzed azide-alkyne Huisgen cycloaddition. Next a donor dye, Cy3-hydrazide, was added to the reaction mixture for conjugation between the keto and hydrazide groups. Dye-labeled CaM was separated from unreacted free dyes using a gel filtration column. Each dye labeling gives rise to an increase in the size of CaM around 0.6–0.7 kDa. Thus, the labeled CaM exhibited a discrete size increase on 15% SDS-PAGE gel when compared to an unlabeled CaM (Figure 3). Dual-labeled CaM (CaM-Cy3-Cy5)

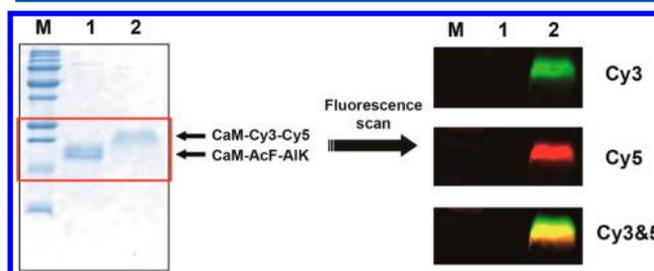


Figure 3. Site-specific dual labeling of CaM. Fluorescence scans of CaM variants: lane 1, CaM-AcF-AIK; lane 2, CaM-Cy3-Cy5.

is apparently the major species present in the labeled mixture. On the basis of the extinction coefficients of Cy3 and Cy5, the dye to protein (D/P) ratio was estimated to be 0.7 and 0.8 for Cy3 and Cy5, respectively, which is higher than our previous bio-orthogonal labeling efficiency.¹⁰ We further confirmed dual labeling of CaM-Cy3-Cy5 using a fluorescence scanner. CaM-Cy3-Cy5 clearly displayed two distinct fluorescent emissions (green for Cy3 and red for Cy5) when the dyes were separately excited (Figure 3), illustrating successful dual labeling.

Our previous dual-labeling system employed site-specific dye conjugation through genetic incorporation of *p*-azidophenylalanine and subsequent bio-orthogonal cross-linking with Cy5-alkyne,¹⁰ but conventional cysteine-maleimide chemistry was applied for the conjugation of a second dye (Cy3). Due to limitations of cysteine-based conjugation, we had to choose

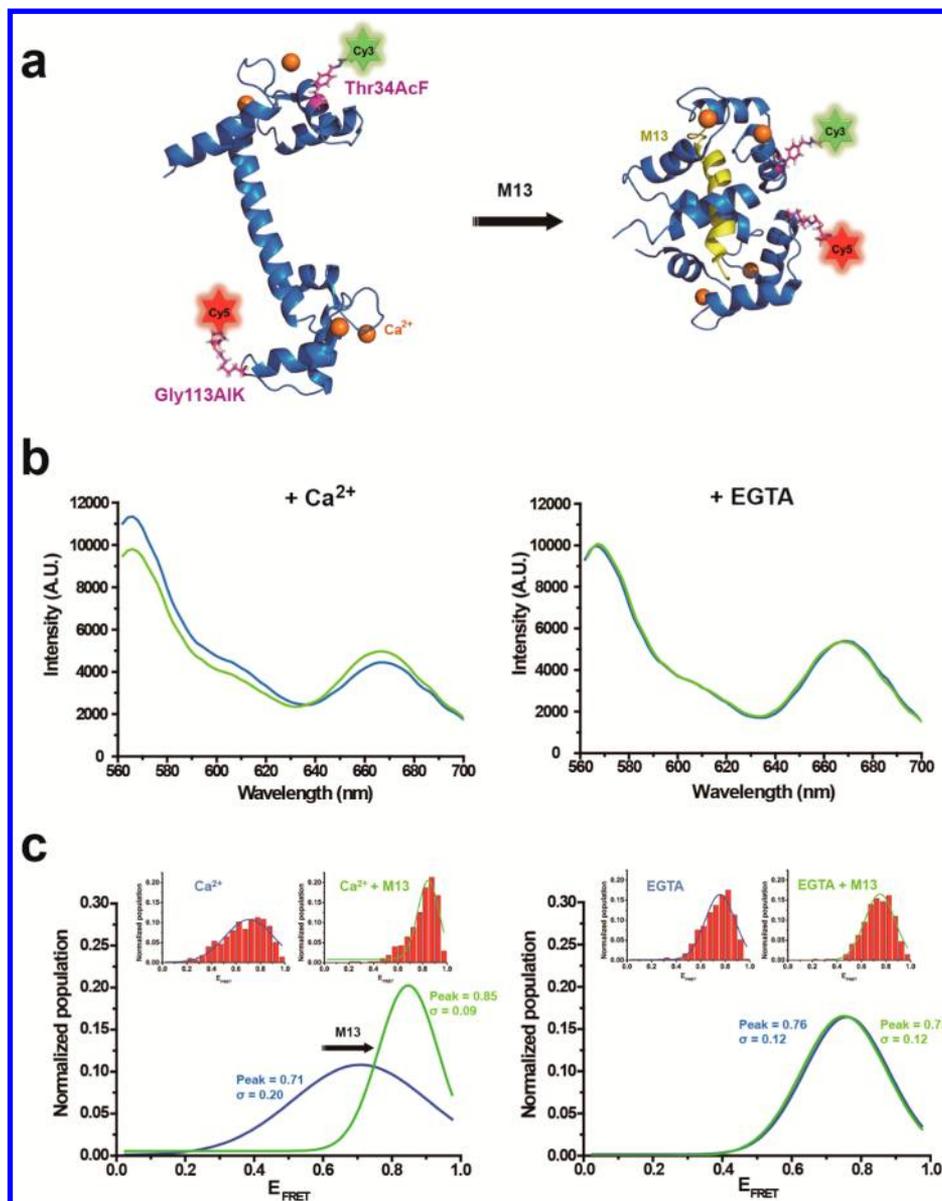


Figure 4. FRET analysis using dual-labeled CaM. (a) Conformational change between CaM-Ca²⁺ and CaM-Ca²⁺-M13 complexes. (b) Ensemble FRET and (c) smFRET experiments using CaM-Cy3-Cy5. The conformational change in CaM-Ca²⁺ was induced by the M13 peptide (10 μM).

maltose binding protein (MBP) as a model protein for smFRET study, because it does not contain any cysteine residue. However, since our current dual-labeling strategy employs two different bio-orthogonal conjugations, it is free of cysteine-related problems, and thus, it can be applied to a wide range of proteins of interest for molecular dynamics study.

Ensemble and Single-Molecule FRET Analysis of Dual-Labeled Calmodulin. To demonstrate the utility of our labeling strategy, we first performed ensemble FRET experiments using double-labeled CaM (CaM-Cy3-Cy5). The conformational change of CaM was induced in response to the peptide M13 in the presence of Ca²⁺ ion (Figure 4a), and the FRET signal (fluorescence emission spectrum of the acceptor dye) was measured under 532 nm excitation for the donor dye. In the presence of M13 peptide, both a decrease in the Cy3 emission intensity (signal at 570 nm) and an increase in the Cy5 emission intensity (signal at 670 nm) were detected, which demonstrates the ligand-binding properties of the dual-labeled CaM (Figure 4b). When calcium binding to CaM was

hindered by EGTA, no fluorescence signal change was detected as expected (Figure 4b).

For analysis of the conformational change, we also attempted smFRET analysis of CaM-Cy3-Cy5. To this end, CaM-Cy3-Cy5 was fused with biotin acceptor peptide (BAP) at the N-terminus and attached with biotin²² through *in vitro* biotinylation with a yield of 85% based on a HABA/avidin assay (Figure S-3 in the Supporting Information). The resulting CaM-Cy3-Cy5 was immobilized on a PEG-based biotin-coated surface via specific interaction between neutravidin and biotin.¹⁰ FRET efficiencies (E_{FRET}) were obtained by measuring the fluorescence intensities of individual CaM molecules in the imaging area of the total internal reflection fluorescence (TIRF) microscope (45 × 90 μm²). SmFRET experiments with CaM-Cy3-Cy5 in the presence of a Ca²⁺ ion exhibited a broad population distribution and yielded a peak with an observed E_{FRET} of 0.71 in the histogram when Cy3 was excited (Figure 4c). Experiments under the same conditions except for additional M13 peptide showed a large shift in population

distribution toward the high E_{FRET} state and produced a sharper Gaussian peak with an observed E_{FRET} of 0.85 (Figure 4c), demonstrating the conformational change in the CaM-Ca²⁺ complex upon binding to the M13 peptide. When it comes to the amplitude of the M13 binding effect, the E_{FRET} shift by the ligand in the single-molecule measurements is more dramatic than the acceptor emission increase by the ligand in the ensemble measurements. SmFRET analysis is considered more accurate and sensitive than ensemble-averaged examination because a homogeneously labeled population is sorted and each molecule is individually analyzed.²³ Experiments with EGTA instead of Ca²⁺ ion displayed no change in both distribution of population and E_{FRET} values (Figure 4c), indicating little conformational change in Ca²⁺-free CaM by the M13 peptide as expected.

CONCLUSIONS

In summary, we have developed a simple and general approach to site-specific dual labeling of proteins. The present strategy employs readily available UAAs (*p*-acetylphenylalanine and alkynyllysine), commercial dye conjugates, and the engineered aminoacyl-tRNA synthetase/tRNA pairs (AcFRS/tRNA_{CUA} and AIKRS/tRNA_{UCA}). Our method enables highly selective bio-orthogonal conjugation of two fluorescence dyes, being devoid of problems and limitations of conventional cysteine-based labeling; thus, it can be generally applied to various proteins for FRET analysis. Our labeling scheme can be extended to site-specific multiple labeling of proteins, which will be useful for investigating more complicated molecular dynamics.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. 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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