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LETTER

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Efficient Single-Molecule Fluorescence Resonance Energy Transfer Analysis by Site-Specific Dual-Labeling of Protein Using an Unnatural Amino Acid

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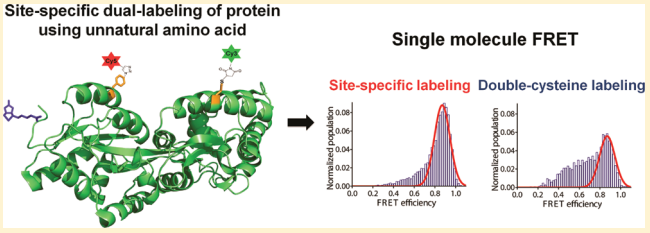
Supporting Information

ABSTRACT: Single-molecule fluorescence resonance energy transfer (smFRET) measurement provides a unique and powerful approach to understand complex biological processes including conformational and structural dynamics of individual biomolecules. For effective smFRET analysis of protein, site-specific dual-labeling with two fluorophores as an energy donor and an acceptor is crucial. Here we demonstrate that site-specific dual-labeling of protein via incorporation of unnatural amino acid provides a clearer picture for the folded and unfolded states of the protein in smFRET analysis than conventional labeling using double cysteines. As a model study, maltose-binding protein (MBP) was dually labeled via incorporation of ρ-azido-L-phenylalanine and cysteine at specific positions, immobilized on a surface, and subjected to smFRET analysis under native and denaturing conditions. The resulting histograms show that site-specific dual-labeling results in a more homogeneous distribution in protein populations, enabling a precise smFRET analysis of protein.



Single-molecule fluorescence resonance energy transfer (smFRET) analysis provides a variety of information including conformational and structural dynamics and localized or colocalized position of the tagged protein.[1−5] When two fluorophores are used as an energy donor and an energy acceptor, which constitute a pair for fluorescence resonance energy transfer (FRET), smFRET measurements convey dynamic information occurring on a few nanometer scales, entailing successive changes in the interdye distance, which are finally translated into the real-time change of the FRET efficiency between two fluorophores.[1] In addition, when dually labeled molecules are immobilized on the surface, the FRET efficiency is recorded while the molecular conditions are varied via microfluidic buffer exchange. This experimental setup allows the study of the structural and conformational dynamics of the same protein molecules under different molecular conditions, such as unfolding and refolding pathways.[2−5] The smFRET analysis of a surface-immobilized protein has proven to be much more practical and widely applicable than initially anticipated because external mechanical vibrations, which would be destructive for a force spectroscopy, usually agitate two fluorophores in an identical way and thus leave their intermolecular distance largely unaffected.

Despite remarkable advances in smFRET measurements, site-specific labeling of protein with the donor and acceptor fluorophores remains a challenge. Introducing two cysteine residues to the desired labeling sites is the most conventional strategy, but this scheme has no labeling selectivity, thus suffering from the heterogeneity in dye labeling. One fluorophore can be attached to the first cysteine and the other to the second cysteine, and the opposite configuration is equally probable. As an approach to site-specific labeling of protein, incorporation of a variety of unnatural amino acids at specific position followed by conjugation with a fluorophore was developed, and notable successes have been reported.[6−8] The use of unnatural amino acids was shown to be effective for solving the heterogeneity in dye labeling. One immediate and general question is if such site-specific labeling schemes with unnatural amino acids have any real, practical advantages in smFRET analysis compared to the conventional method with double cysteine residues.

Here, we demonstrate that site-specific labeling of protein via incorporation of unnatural amino acid provides a clearer picture for the folded and unfolded states of the protein than the usual double-cysteine mutant. With the use of maltose-binding protein (MBP) as a model system, ρ-azido-L-phenylalanine and cysteine were site-specifically incorporated and labeled using Cy5 and Cy3

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dyes, respectively. The resulting MBP (UC-MBP) was immobilized on a surface and subjected to smFRET analysis for the conformational change with the use of guanidine chloride (GdHCl) as the denaturant. For comparison, we also tested MBP labeled using two cysteine residues (CC-MBP).

EXPERIMENTAL SECTION

Materials. ρ-Azido-L-phenylalanine was purchased from Bachem. Maleimide-Cy3 and NHS ester-Cy5 dyes were purchased from GE Healthcare. HABA/avidin reagent was purchased from Sigma.

^{1}H −NMR and LC/MS. ^{1}H NMR spectrum was recorded on a Varian Inova (600 MHz) spectrometer using solvent residues as reference. LC/MS was conducted using a mass spectrometer (Agilent 1100 + G1958, Agilent Technologies, Inc.).

Synthesis of Cy5-alkyne. To a solution of *N*-methylmorpholine (3.2 μL, 30 μmol) and propargylamine (1.2 μL, 19 μmol) in 0.32 mL of DMF was added Cy5Mono NHS ester (10 mg, 13 μmol). After stirring for 18 h at room temperature in the dark, the solution was purified using column chromatography on silica gel eluting with 20% MeOH in dichloromethane to give Cy5-alkyne (9.6 mg, 13 μmol): ^{1}H NMR (600 MHz, CD_3OD) δ 8.30 (tt, J_1 = 13.2 Hz, J_2 = 4.2 Hz, 2H), 7.88−7.85 (m, 4H), 7.32 (t, J = 8.4 Hz, 2H), 6.67 (t, J = 12.0 Hz, 1H), 6.33 (dd, J_1 = 13.2 Hz, J_2 = 12.0 Hz, 2H), 4.16 (q, J = 7.2 Hz, 2H), 4.10 (t, J = 7.2 Hz, 2H), 3.90 (d, J = 2.4 Hz, 2H), 2.55 (t, J = 2.4 Hz, 2H), 2.19 (t, J = 7.2 Hz, 2H), 1.81 (m, 2H), 1.73 (s, 12 H), 1.67 (m, 2H), 1.43 (m, 2H), 1.37 (t, J = 7.2 Hz, 3H); LC/MS m/z 692 ($M - H^-$), 714 ($M + Na^+ - 2H^-$).

Cloning and Mutagenesis for Incorporation of Unnatural Amino Acid. A gene coding for maltose-binding protein (MBP) was cloned from the pMAL-p2X vector. Biotin acceptor peptide (15 residues) gene of BirA was attached at the N terminal of the MBP gene and then inserted between *NdeI* and *XhoI* restriction sites of plasmid pET21a. The resulting MBP gene had a hexahistidine tag at the C terminal. For the construction of double mutated MBP, Lys42 and Lys370 were changed to an amber codon (TAG) or cysteine. Engineered *Methanococcus jannaschi* tyrosyl-tRNA synthetase (*Mj*TyrRS)[9] and biotin-protein ligase (BirA) genes from pBIRAcm plasmid (Avidity) were inserted into pRSF-Duet-1 (Navagen), resulting in pRSF-*Mj*TyrRS-BirA. The *Methanococcus jannaschi* tyrosyl-$tRNA_{CUA}$ (*Mj*$tRNA_{CUA}$) gene[9] was inserted into the pACYC184 vector (New England Biolabs) instead of the tetracycline resistance gene, and the resulting construct was designated pTech-*Mj*$tRNA_{CUA}$.

Protein Expression and Purification. To express MBP with an unnatural amino acid, pET21a-MBP was transformed into *E. coli* BL21 (DE3) cells with plasmids pRSF-*Mj*TyrRS-BirA and pTech-*Mj*$tRNA_{CUA}$. The cells were cultivated in M9 minimal media containing 1% glycerol, 100 μg/mL carbenicillin, 50 μg/mL kanamycin, 50 μg/mL chloramphenicol, and 50 μM d-biotin. Cells were grown at 37 °C until OD_{600} reached around 0.6, followed by addition of 0.2 mM IPTG and 1 mM ρ-azido-L-phenylalanine. After cultivation at 30 °C for 20 h, cells were harvested and lysed. The cell lysate was clarified by centrifugation at 16 000*g* at 4 °C for 30 min and filtered. Filtrated crude extract was mixed with 500 μL of Ni-NTA superflow (Qiagen) and incubated with agitation for 1 h at 4 °C. MBP was eluted using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), and purified MBP was desalted with PD-10 desalting column (GE Healthcare, Sephadex G25 Medium) using PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) containing 5% glycerol. Protein concentration was measured by the Bradford method. HABA/avidin reagent (Sigma) was used to quantify biotin-conjugated protein.

Mass Spectrometry. Purified MBP was tryptic digested in 0.1 M Tris-HCl (pH 8.0) containing 5 mM DTT at 37 °C for 16 h. The digested protein solution was analyzed using a tandem LC/MS spectrometer (QTOF II, Micromass, Manchester, U.K.). A sample solution was loaded onto a guard column (300 μm × 5 mm) packed with C18 (100 Å pore size, 5 μm diameter) using a FAMOS autosampler (LC packings, Sunnyvale, CA), and peptide fragments were separated on a silica microcapillary reversed phase column (75 μm × 10 cm) using a Ultimate (LC packings, Sunnyvale, CA). The mobile phases, A and B, contained 0.5% acetic acid and 0.02% formic acid and each was composed of 0% and 80% ACN, respectively. The gradient began at 5% B for 20 min and was ramped to 20% B for 3 min, to 60% B for 30 min, followed by 100% B for 2 min and then was held at 100% B for 7 min, and finally was dropped to 5% B for 2 min. The column was equilibrated with 5% B for 9 min before the next run. During gradient elution, 3 MS/MS spectra were acquired in a data-dependent tandem mass spectrometry (MS/MS) mode from a MS master spectrum. A threshold of 10 (counts/s) was required to trigger an MS/MS event. The electrospray voltage was set to 2.5 kV, and the specific m/z value of the peptide fragmented by collision induced dissociation (CID) was excluded from reanalysis for 100 s.

Dye Labeling. Cy5-alkyne dye was first conjugated with an azide group of ρ-azido-L-phenylalanine-containing MBP using click chemistry as described elsewhere[10−14] with minor modification. Briefly, 250 μM Cy5-alkyne was mixed with protein solution (25 μM) containing 1 mM $CuSO_4$ and 1 mM TBTA (tri(1-benzyl-[1,2,3]-triazol-4-ylmethyl)amine), followed by addition of 1 mM sodium ascorbate. The mixture was incubated in the dark at 4 °C for 16 h. The resulting MBP was desalted with PD-10 desalting column using PBS containing 5% glycerol for removal of unreacted free dye. The dye to protein ratio (Cy5/MBP) was determined by measuring the absorbance at 280 and 650 nm for MBP and Cy5, respectively. Cy5-labeled MBP was consecutively labeled with maleimide-Cy3 dye according to the supplier's manual. Dual-labeled MBP was again desalted with a PD-10 column using PBS containing 5% glycerol. The dye to protein ratio (Cy3/MBP) was estimated by measuring the absorbance at 280 and 552 nm for MBP and Cy3, respectively. Extinction coefficients of MBP (72 000 M^{-1} cm^{-1} at 280 nm), Cy5 (250 000 M^{-1} cm^{-1} at 650 nm), and Cy3 (150 000 M^{-1} cm^{-1} at 552 nm) were used to calculate molar concentration of the protein and the dyes.

Fluorescence Scanning. Dual-labeled MBPs were loaded on 10% SDS PAGE and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad). The membrane was scanned for Cy3 and Cy5 using a scanner (GenePix Persnal 4100A, HP).

Circular Dichroism (CD) Analysis. For the CD analysis of native and double mutated MBPs, protein solution was loaded into a 0.1 mm quartz cuvette, and CD spectra were obtained on a JASCO J-815. CD spectra were taken between 250 and 200 nm with a 1 nm increment, and a background spectrum with free buffer solution was subtracted. For the analysis of the unfolding profile, native and double mutated MBPs or neutravidin were

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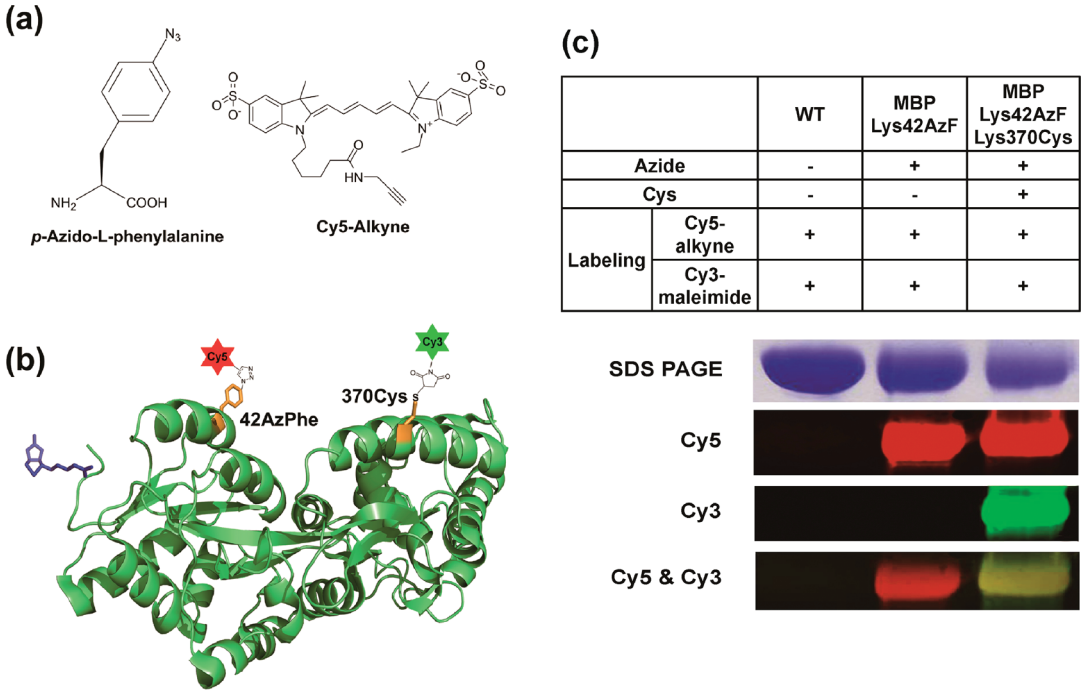

Figure 1. Site-specific dual-labeling of protein via incorporation of unnatural amino acid and click chemistry. (a) Structures of ρ-azido-L-phenylalanine and Cy5-alkyne. (b) Site-specific dual-labeling of MBP. Biotin (blue) is attached at the N-terminal of MBP for surface immobilization. (c) Fluorescence images of site-specifically dual-labeled MBP.

diluted to a final concentration of 0.7 mg/mL with PBS buffer in the presence of a predetermined concentration of guanidine chloride (GdHCl) and then CD spectra were taken. CD values at 222 nm were plotted at different GdHCl concentrations.

Single-Molecule FRET Experiments. Quartz slides and glass coverslips were treated with 99:1 (mol/mol) PEG/biotin-PEG (Laysan Bio) to prevent nonspecific binding of proteins. A fluid chamber was made by sandwiching these PEG-coated slide and coverslip with a double sticky tape as a spacer, followed by sealing with epoxy.[15,16] Two holes were drilled on the quartz slide side (before PEG treatment) to be used as the inlet and outlet for solution exchange. The inside surface of the chamber was coated with 0.1 mg/mL neutravidin. Biotinylated, dual-labeled MBP was diluted to a final concentration of 0.5 nM, injected into a sample chamber, and immobilized on the PEG-coated surface via biotin−neutravidin interactions through incubation for 10 min. The channel was washed with a PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) containing 3 mM dithiothreitol (DTT) to remove free proteins and reduce the intermolecular disulfide bond. For unfolding of dual-labeled MBP, a solution containing different concentrations of GdHCl was introduced into the sample chamber, and refolding of the unfolded MBPs was induced by injecting the PBS buffer without GdHCl. During imaging, an imaging buffer (10 nM protocatechuate-3,4-dioxygenase (PCD), 2.5 mM protocatechuic acid (PCA), 2 mM Trolox, 3 mM DTT in PBS buffer)[15] was present in the buffer to reduce photo bleaching and dye blinking. Single-molecule FRET measurements were carried out on a prism type total-internal-reflection fluorescence microscope, which is based on an inverted microscope (IX-71 Olympus). Fluorescence signals from the entire sample plane ($45 \times 90\ \mu m^2$) were collected by a 60× water immersion lens (UPLSAP060XW, Olympus) and separated by a dichroic mirror that had the threshold at 635 nm (Chroma). The two separated rays were focused to form two images of the sample plane on an electron multiplying charged-coupled device camera (iXon DU897v, Andor Technology), namely, the donor- and acceptor-channel images, respectively. A time resolution of 50 ms was used for real time recording. As a result, signals of one single molecule appear as two diffraction-limited point spread spots in the donor and acceptor channels, respectively. These two diffraction limited spots of the same single molecule were identified by a custom-made program written in IDL (Research systems), and time trajectories of fluorescence intensities in the donor (I_D) and acceptor (I_A) channels were generated. The equation of $I_A/(I_A + I_D)$ was used to determine the FRET efficiency.[5] The average fluorescence intensities from the donor and acceptor channels measured before immobilization of the sample were considered as the background fluorescence and subtracted uniformly from the fluorescence intensities from individual proteins. The leakages of donor fluorescence into the acceptor channel and that of acceptor fluorescence into the donor channel were also taken into account.

RESULTS AND DISCUSSION

Incorporation of Unnatural Amino Acid. We attempted to incorporate ρ-azido-L-phenylalanine (AzPhe, Figure 1a) into maltose-binding protein (MBP) using orthogonal *Methanococcus jannaschi* tyrosyl-tRNA synthetase (*Mj*TyrRS)/$tRNA_{CUA}$ mutant pair for site-specific labeling of MBP with Cy5-alkyne using click chemistry. *In vivo* incorporation of unnatural amino acids containing azides or alkynes into protein was reported to be a simple and practical method of tagging proteins with functional groups for conjugation.[12−14] It was shown that AzPhe can be incorporated site-specifically into protein using an amber codon

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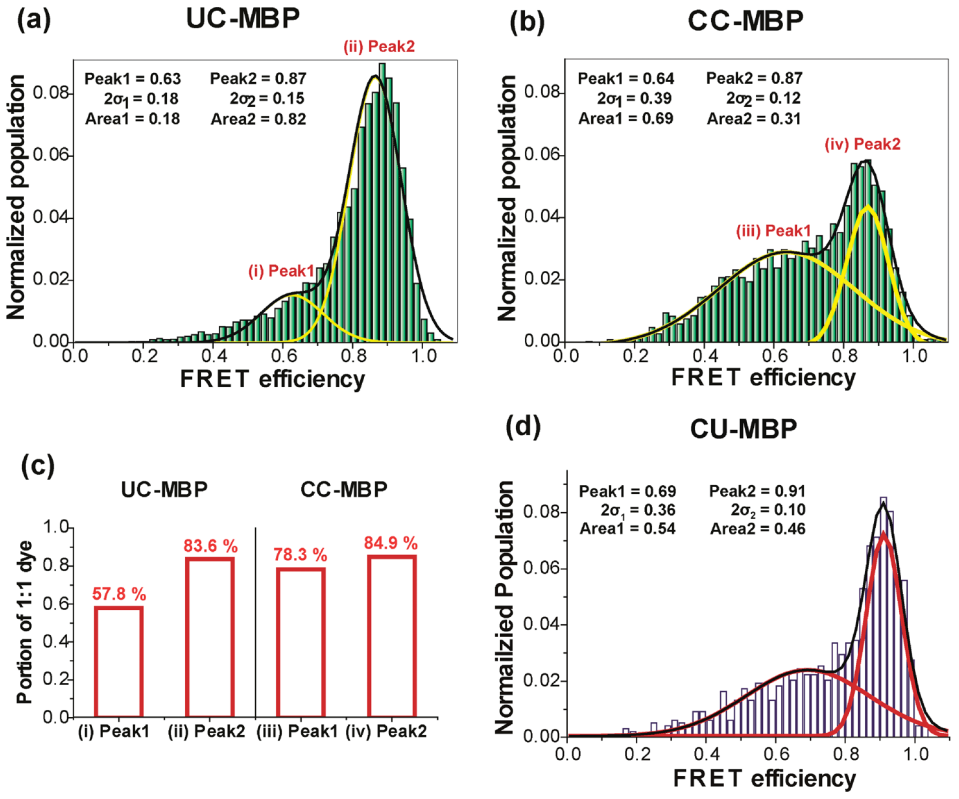

Figure 2. FRET efficiency histograms of dual-labeled MBPs under native conditions. (a, b) UC-MBP and CC-MBP without GdHCl was described with double Gaussian distribution. (c) The portion of a single pair of dyes in UC-MBP and CC-MBP was confirmed by observing the photobleaching of donor and acceptor using real-time traces (as shown in Figure S-5 in the Supporting Information). (d) FRET efficiency histogram of CU-MBP under native conditions. The labeling positions of Cy3 and Cy5 were exchanged when compared with those of previously constructed UC-MBP.

(TAG) using an engineered orthogonal AzPheRS/$tRNA_{CUA}$ pair in *E. coli*.[9] Azides are commonly used with alkyne for click chemistry, namely, Cu-catalyzed azide−alkyne cycloaddition, and the reaction was shown to be quantitative, robust, easy-to-use, and bio-orthogonal under physiological conditions.[10,17]

For smFRET analysis of MBP under native and denaturing conditions, we selected two residues Lys42 and Lys370 for dual-labeling of MBP with Cy5-alkyne and Cy3-maleimide (Figure 1b), based on the crystal structure of MBP (PDB ID, 1LLS). They are located outside loop regions, and the distance between Cα of two residues (3.21 nm) seems appropriate for FRET analysis. An amber codon (TAG) was inserted at the position Lys42 for incorporation of AzPhe, and Lys370 was changed to cysteine. For immobilization of MBP via biotin−avidin interaction on a slide glass, a gene for biotin acceptor peptide was attached directly to the N-terminal of MBP using the method described elsewhere,[18] and the resulting construct was coexpressed with BirA protein (biotin-protein ligase of *E. coli*) for the biotinylation. Biotin content was estimated to be 60−70% in MBP population by a HABA/avidin assay. Expression level of AzPhe-containing MBP (AzF-MBP) was about 15% of total proteins in *E. coli*, and incorporation of AzPhe was confirmed by LC−MS/MS analysis after tryptic digestion (Figure S-1 in the Supporting Information). For comparison of site-specific dual-labeling with double cysteine labeling in smFRET analysis, both Lys42 and Lys370 of MBP were changed to cysteine (Cys-MBP). Double mutated MBPs (AzF-MBP and Cys-MBP) were purified, and their biochemical properties were compared with that of native MBP in terms of unfolding profile and secondary structures. As a result, double mutated MBPs showed nearly the same properties as native one (Figure S-2 in the Supporting Information).

Site-Specific Dual-Labeling of MBP. For labeling of MBP with the acceptor onto the azide group of AzPhe, Cy5-alkyne (Figure 1a) was first synthesized using commercially available Cy5-mono NHS ester (Figure S-3 in the Supporting Information). Cy5-alkyne was purified and subjected to incubation with AzF-MBP in the presence of 1 mM $CuSO_4$, Na-ascorbate, and TBTA in PBS buffer (pH 7.4) as described elsewhere.[10−14] Cy5-labeled MBP (MBP-Cy5) was separated from unreacted free dye through the gel filtration, and the dye to protein (D/P) ratio was estimated to be 0.66. For dual-labeling, MBP-Cy5 containing a single cysteine was further reacted with maleimide-modified Cy3 dye (UC-MBP). We checked orthogonal reactivity of functional groups in MBP with Cy3 and Cy5 dyes using a fluorescence scanner. As shown in Figure 1c, only MBP with two functional groups displayed distinct fluorescent colors when scanned for Cy3 and Cy5 dyes while MBP containing sole azide moiety showed only Cy5 fluorescence after same dual-labeling procedures, confirming site-specific labeling with Cy3 and Cy5 dyes. No cross-reactivity between azide and cysteine for Cy5-alkyne and Cy3-maleimide was observed, which indicates that orthogonal dual-labeling was achieved through two unique reactive groups on MBP. At the same time, Cys-MBP was labeled using a mixture of maleimide-modified Cy3 and Cy5 dyes. Separation and estimation of dye contents in the resulting MBP (CC-MBP) were also followed using the same procedures as for UC-MBP.

Single-Molecule FRET Analysis of MBP. In order to check whether labeling of MBP via incorporation of unnatural amino

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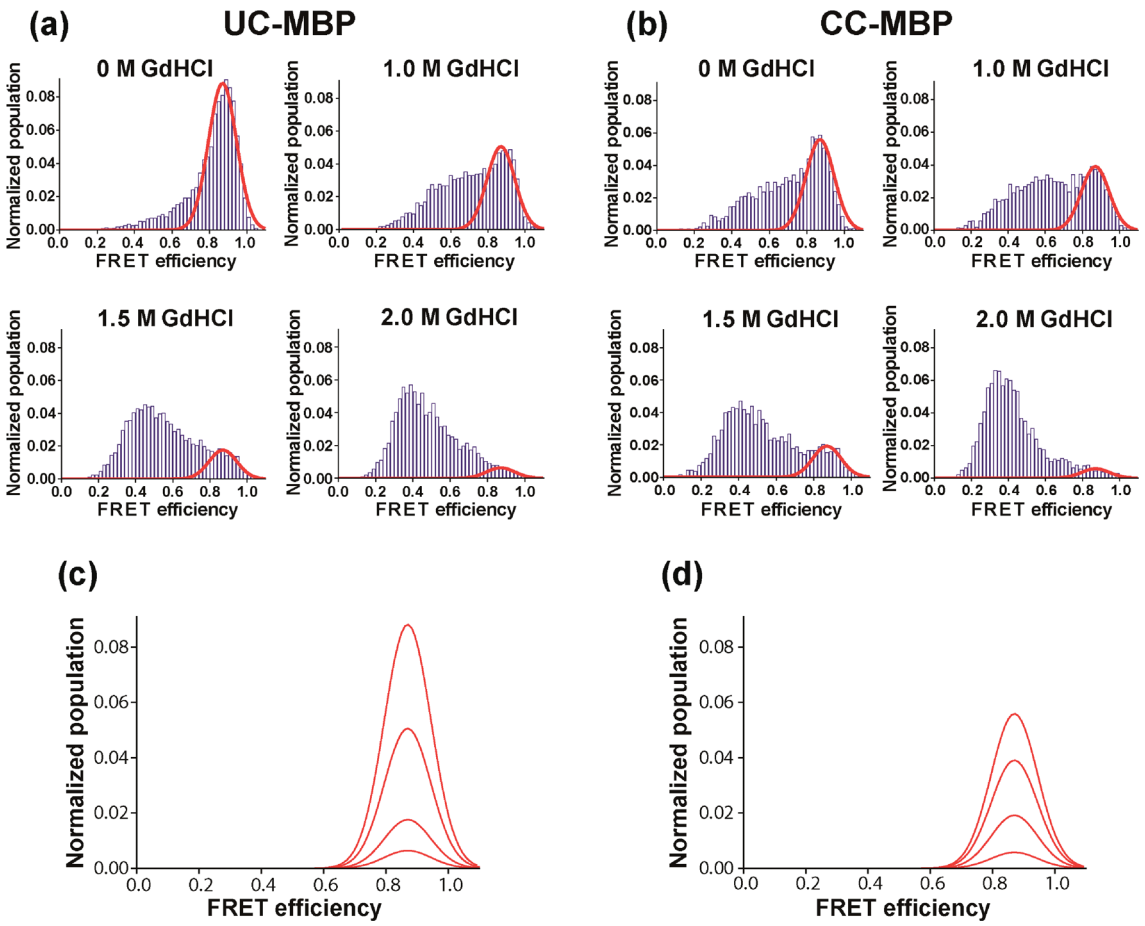

Figure 3. Histograms of smFRET analysis. FRET efficiency histograms of UC-MBP (a) and CC-MBP (b) under native and denaturing conditions are compared. Relative populations of the folded fraction that is under the red Gaussian curve of E_{FRET} = 0.87 are marked, and the changes of the relative populations are compared between UC-MBP (c) and CC-MBP (d).

acid results in any difference in smFRET compared to that using conventional double cysteines, we attempted a smFRET analysis of dual-labeled MBPs. The UC-MBP and CC-MBP were immobilized on a PEG polymer cushion via the specific interaction between neutravidin and biotin. A few nanomolar concentration of MBP was used, and a few hundreds of fluorescent MBP molecules were observed in one imaging area of the TIRF microscopy (45 × 90 μm^2). With an electron-multiplying CCD, the fluorescence intensities of individual protein molecules in the same imaging area could be recorded in a parallel manner with the time resolution of 50 ms (Figure S-4 in the Supporting Information). The donor (I_D) and acceptor (I_A) fluorescence intensities from each single molecule were measured, and the FRET efficiencies (E_{FRET}) were calculated using the intensity-based equation; $I_A/(I_A + I_D)$.

The surface-immobilized MBPs were subjected to smFRET measurements under native and denaturing conditions. We first investigated the histograms of FRET efficiencies for UC-MBP and CC-MBP under native conditions (Figure 2). Without GdHCl, UC-MBP displayed a sharp Gaussian peak at E_{FRET} = 0.87 ± 0.08 (Figure 2a), as predicted by the crystal structure (PDB ID, 1LLS; expected E_{FRET} based on the Cα of two residues (3.2 nm) is 0.96 when the Föster radius of Cy3 and Cy5 is assumed to be 5.4 nm), and the relative population of this peak was estimated to be 0.83 (population in Gaussian curve as Figure 3a). The UC-MBP thus seems to maintain its intact structure even after dye labeling and surface immobilization. In contrast, CC-MBP, which is labeled via two cysteine residues at the same positions, shows a much broader distribution of E_{FRET}. Under the native conditions, CC-MBP also shows the high-FRET peak at 0.87 as UC-MBP, but the relative population of this peak is only 0.53 (population in Gaussian curve as Figure 3b). Furthermore, CC-MBP displays a larger population in the middle FRET range, which was fitted by a broad Gaussian (σ = 0.20) with its peak at E_{FRET} = 0.64 ± 0.20 (Figure 2b). We tested the possibility if this increased the middle FRET population simply results from multiple donor or acceptor dyes due to protein aggregation.[5] By identifying the number of the photobleaching steps in the real-time traces of individual MBP (as shown in Figure S-5 in the Supporting Information), we counted the number of the donor and acceptor dyes attached to individual MBP molecules. When all the real-time traces that started with the E_{FRET} values designated as the middle FRET range (0.64 ± 0.20) were collected, more than 78% of the traces exhibited a single pair of the donor and acceptor dyes (Figure 2c). We tested whether the labeling position of fluorescence dyes at MBP has an effect on the histograms of FRET efficiency. For this, an amber codon (TAG) was inserted at position Lys370 for incorporation of AzPhe and Lys42 was mutated to cysteine (CU-MBP). Hence, the labeling positions of Cy3 and Cy5 were exchanged compared with those of previously constructed MBP. We conducted a smFRET

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analysis of CU-MBP according to the procedure as described above. As a result, although there was a little shift of peak1 and peak2 with slightly increased middle FRET population (Figure 2d), heterogeneity in dye labeling is not a dominant factor. This result indicates that the increased middle FRET range reflects some internal changes in single MBP molecules, which become pronounced when the double cysteine residues were used for labeling with fluorescent dyes.

Homogeneous distributions of dual-labeled protein as a native structure, as shown in Figure 2, is a critical point for precise and detailed observation in smFRET analysis to see the changes of intermolecular distance. In order to see further differences in FRET behavior between UC-MBP and CC-MBP, we performed a smFRET analysis for the unfolding process under denaturing conditions of UC-MBP and CC-MBP at different GdHCl concentrations and compared the distributions of the FRET efficiency (Figure 3). As the GdHCl concentration increased to 1 M, E_{FRET} of UC-MBP exhibited a much broader distribution spanning the middle E_{FRET} values over 0.4−0.8 (Figure 3a). When the GdHCl concentration was finally raised to 2 M for UC-MBP, the E_{FRET} values were largely shifted to a low efficiency region centered around 0.37. In the case of CC-MBP, a high-FRET peak at 0.87 reduced first at a 1 M GdHCl concentration (Figure 3b). As the GdHCl concentration was further increased to 2 M, both high- and middle FRET populations were shifted to the unfolded state at E_{FRET} = 0.37, showing a similar pattern to that observed with the UC-MBP. This convergence of the FRET values under highly denaturing condition indicates that the fluorescence characteristics of the dyes are not different for UC-MBP and CC-MBP, which supports that the disparate FRET pattern between UC-MBP and CC-MBP resulted from the internal changes in the MBPs. Neutravidin, which was used for immobilization of MBP, was shown to remain stable up to 3.5 M GdHCl (Figure S-6 in the Supporting Information). Changes of the relative population in smFRET analysis can be deciphered as conformational changes (unfolding profile) of the protein. Summarized changes of population in high E_{FRET} (0.87), predicted as native status, according to the concentration of denaturant present a striking contrast between UC-MBP (Figure 3c) and CC-MBP (Figure 3d). High homogeneity of dual-labeled proteins through site-specific incorporation of unnatural amino acid is expected to have practical advantages in the smFRET study of proteins. In view of the outcomes so far achieved, our results strongly imply that site-specific dual-labeling by incorporation of unnatural amino acid enables distinct dissection of the unfolded and folded states of MBP, allowing more sensitive analysis of the conformational change of the protein. Although a lot of smFRET experiments using the double-cysteine labeling method have provided successful results to understand biomolecular dynamics and mechanisms, site-specific dual-labeling using an unnatural amino acid might offer a chance to see more definite pictures in smFRET analysis of proteins.

CONCLUSIONS

We have shown that the site-specific labeling of protein via incorporation of an unnautural amino acid provides a greater contrast between the folded and unfolded states of the protein in smFRET analysis than the conventional labeling using double cysteines. Site-specifically dual-labeled UC-MBP displayed homogeneous distributions in the smFRET histogram of the native state, whereas the CC-MBP generated unexpected broad distributions. The homogeneity of UC-MBP enabled more delicate analysis of the unfolding profiles of the protein. Considering this case of MBP, site-specific dual-labeling of the protein might offer a new chance for more precise smFRET analysis of the protein.

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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