

Incorporation of Non-Natural Amino Acids with Two Labeling Groups into the N-Terminus of Proteins

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Incorporation of non-natural amino acids into proteins is a useful method for protein researches, including position-specific labeling with fluorophores and biotin. Here, we incorporated single non-natural amino acids with both fluorophore and biotin into the N-terminus of proteins in a cell-free translation system. α-Biotinyl-*p*-(BODIPYFL-amino)phenylalanine derivatives, with or without an aminohexyl linker between the biotin moiety, were synthesized and attached to an *Escherichia coli* initiator tRNA that had a CUA anticodon. The aminoacylated initiator tRNA was added to an *E. coli* cell-free translation system together with an mRNA encoding maltose binding protein that had a UAG initiator codon. Fluorescence analysis of SDS-PAGE, Western blot analysis, and mass spectrometry demonstrated that the biotinylated BODIPYFL-aminophenylalanine derivatives were successfully incorporated into the N-terminus of the protein, although the aminohexyl linker slightly decreased the incorporation efficiency. Fluorescence spectroscopy measurements indicated that complexation with streptavidin significantly quenched the fluorescence of BODIPYFL, and the aminohexyl linker facilitated the fluorescence quenching. The biotinylated BODIPYFL-aminophenylalanine was also used for double labeling several proteins. This method is a general and useful tool for the N-terminal-specific modification of proteins with two moieties.

Site-specific incorporation of chemically synthesized non-natural amino acids into proteins in cell-free and in-cell translation systems is a useful method for the analysis of the structure and function of proteins and for protein engineering. Using this method, a variety of non-natural amino acids, including those containing fluorophores or biotin, have been incorporated into specific internal positions of proteins in response to a UAG codon or four-base codons.[1–7] However, some non-natural amino acids cannot be incorporated through the translation elongation process because of the limited substrate specificity of the ribosomal system. We have shown that aromatic non-natural amino acids are incorporated into proteins in a structure-dependent manner, and *p*-substituted phenylalanine derivatives are good substrates in the translation elongation process.[8] According to this fact, *p*-aminophenylalanine derivatives containing fluorophores and biotin have been developed for position-specific protein labeling.[2,3] The labeled amino acids can be doubly incorporated into proteins in response to two different four-base codons[2] or a combination of four-base codon and a amber codon.[9] Nonetheless, to date, the introduction of single non-natural amino acids containing two labeling groups in the translation elongation process has not been achieved, possibly because of their large molecular size. Incorporation of double-labeled amino acids will simplify the preparation of double-labeled proteins using the non-natural amino acid mutagenesis method.

In contrast, the N-terminal-specific incorporation of amino acid derivatives through the expansion of the translation initiation process has been developed. Previous studies have reported that amino acid derivatives, other than methionine,[10–13] and initiator codons, other than AUG,[14,15] are accepted in the translation initiation process. We have shown that carboxylic acids without an α-amino group can be incorporated in response to an expanded initiator codon UAG.[16] These studies suggest that the translation initiation process is potentially very flexible and available as a useful tool to introduce non-natural amino acids, which are difficult to incorporate into proteins, through the translation elongation process. In addition, the N-terminus is a desirable position for protein modification, particularly for protein labeling with fluorophores and biotin, because in most cases the N-terminus does not contribute to protein functions such as catalytic activity and substrate binding.

In this study, we incorporated single non-natural amino acids containing two labeling groups into the N-terminus of proteins through the initiation process in a cell-free translation system (Figure 1a). For this purpose, *p*-(BODIPYFL-amino)phenylalanine (BFLAF) derivatives with biotin at the α-amino group, with or without aminohexyl linkers, were designed and synthesized (BioBFLAF, BioXBFLAF, and BioXXBFLAF, Figure 1b). BFLAF was chosen as the key compound because it has been successfully incorporated into the N-terminus of proteins in response to a UAG initiator codon[16] and it has a free α-amino group that can be selectively linked with biotin. The biotinylated BFLAF derivatives were used to produce proteins containing biotin and fluorophores at the N-terminus in a quantitative manner. The double-labeled proteins will be useful for oriented immobilization of fluorescently labeled proteins onto streptavidin-coated solid phase. The binding of the double-labeled proteins with streptavidin and the fluorescence properties of the streptavidin complexes were also investigated.

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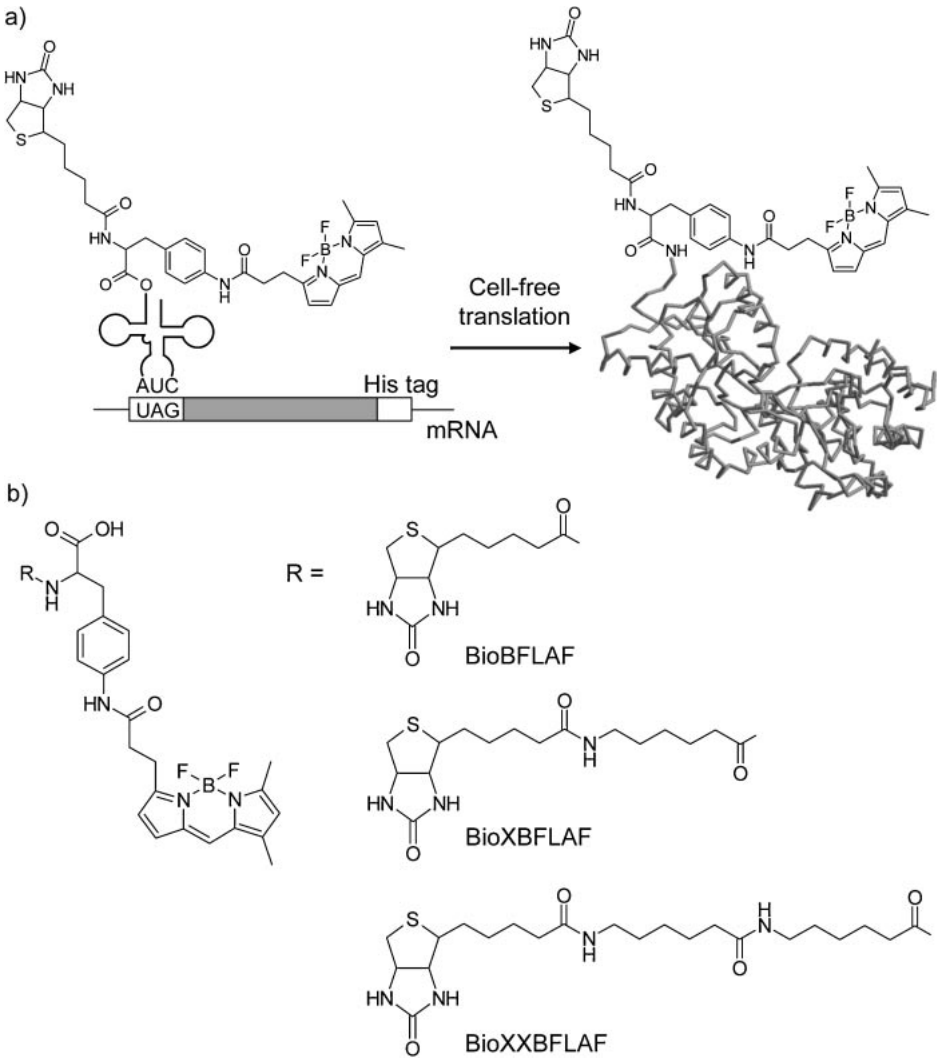

Figure 1. (a) Schematic illustration of the incorporation of non-natural amino acids with two moieties into the N-terminus of proteins in response to a UAG initiator codon. (b) Structures of biotinylated BFLAF derivatives.

Experimental

Materials. Biotin *N*-hydroxysuccinimide esters were purchased from Sigma Aldrich (St. Louis, MO, USA). T4 RNA ligase was from Takara Bio (Otsu, Japan). RTS *E. coli* Linear Template Generation Set was from Roche Diagnostics (Mannheim, Germany). *E. coli* S30 extract for the linear template, alkaline phosphatase-labeled anti-mouse IgG, and MagneHis Ni-particles were from Promega (Madison, WI, USA). Anti-T7 tag antibody and anti-His tag antibody were from Novagen (La Jolla, CA, USA). MicroSpin G25 column was from GE Healthcare (Piscataway, NJ, USA). Streptavidin was from Vector Laboratories (Burlingame, CA, USA). Lysyl endopeptidase was from Wako Pure Chemicals (Osaka, Japan). ZipTip C18 was from Millipore (Bedford, MA, USA).

Synthesis of Biotinylated BFLAF–5′-Phospho-2′-deoxycytidylyl-3′,5′-adenosine (pdCpA). BFLAF–pdCpA was synthesized from aminophenylalanyl–pdCpA and BODIPYFL *N*-hydroxysuccinimide ester as described previously.[2] BFLAF–pdCpA was then biotinylated as follows. 2.2 mM BFLAF–pdCpA in DMSO 50 µL, 50 mM biotin *N*-hydroxysuccinimide ester in DMSO 20 µL, DMSO 30 µL, and aqueous 0.1 M $NaHCO_3$ 10 µL were mixed and incubated on ice for 18 h. The product was analyzed and isolated on a reverse-phase HPLC (Waters XBridge C18, 2.5 µm, $4.6 \times 20\,mm^2$), flow rate 1.5 mL min^{-1} with a linear gradient of 0–100% methanol in 0.38% formic acid, over 10 min. BioXBFLAF– and BioXXBFLAF–pdCpA were similarly synthesized using biotin-X and biotin-XX *N*-hydroxysuccinimide esters, respectively. To determine the yield of the biotinylated BFLAF–pdCpA derivatives, the pdCpA moiety was released from the aminoacyl–pdCpA by alkali-hydrolysis and applied to the HPLC. The peak of pdCpA was quantified using a calibration curve for serial dilutions of standard pdCpA, whose concentration was determined using molar extinction coefficient[17] of $2.3 \times 10^4\,cm^{-1}\,M^{-1}$ at 260 nm. The yields were 53%, 45%, and 54% for BioBFLAF–, BioXBFLAF–, and BioXXBFLAF–pdCpA, respectively. The products were identified by electrospray mass spectrometry (Mariner, Applied Biosystems). BioBFLAF–pdCpA, calculated for $C_{52}H_{63}N_{14}O_{17}P_2SBF_2$ $(M-H)^-$ 1297.3674, found 1297.3651; BioXBFLAF–pdCpA, calculated for $C_{58}H_{74}N_{15}O_{18}P_2SBF_2$ $(M-H)^-$ 1410.4515, found 1410.4562; and BioXXBFLAF–pdCpA, calculated for $C_{64}H_{85}N_{16}O_{19}P_2SBF_2$ $(M-2H)^{2-}$ 761.2644, found 761.2656.

Preparation of Initiator tRNAs Aminoacylated with Bio-

tinylated BFLAF Derivatives. An initiator tRNA with a CUA anticodon and lacking two 3′ nucleotides was prepared as described previously using a PCR primer containing two 2′-*O*-methylated ribonucleotides to suppress an additional single base extension by T7 RNA polymerase.[16] The truncated tRNA was analyzed on a capillary gel electrophoresis (Beckman P/ACE system), and ligated with BioBFLAF–pdCpA derivatives. A reaction mixture (10 μL) contained 5.5 mM HEPES-K (pH 7.5), 15 mM $MgCl_2$, 1 mM ATP, 3.3 mM dithiothreitol (DTT), 2 μg mL^{-1} BSA, 0.25 nmol initiator tRNA lacking 3′ dinucleotide, 0.75 mM BioBFLAF–pdCpA in DMSO 1 μL, and 12 units T4 RNA ligase. The reaction mixture was incubated at 4 °C for 16 h. The tRNA fraction was isolated by ethanol precipitation, and analyzed on HPLC (Applied Biosystems Poros R2/10, 4.6 × 100 mm^2), flow rate 1.0 mL min^{-1} with a linear gradient 0–50% of acetonitrile in 0.1 M triethylammonium acetate (pH 7), over 25 min. The HPLC analysis indicated that 0.18 nmol of aminoacylated tRNA and 0.04 nmol of non-aminoacylated tRNA were obtained, and therefore, 80% of tRNA was aminoacylated. BFLAF–tRNA without the biotin moiety was prepared in a similar manner except that the reaction time was 2 h.

Preparation of a Maltose Binding Protein (MBP) mRNA. A MBP gene containing a TAG initiator codon and a hexahistidine tag (His tag) at the C-terminus was prepared using an RTS *E. coli* Linear Template Generation Set. A 5′ primer (5′-CTTTAAGAAG GAGATATACC TAGAAAATCG AAG-3′) and a 3′ primer (5′-TGATGATGAG AACCCCCCCC AAGCTTATTA GTGGTG-GTGG TGGTGGTGCT TGGTGAT-3′) were used as gene specific primers. A plasmid encoding *E. coli* MBP[3] was used as a template. The resulting DNA fragment was amplified by PCR using a 5′ primer (5′-TCCCCCGGGG GACTAATACG ACTCACTATAG-3′) and the above 3′ primer. The PCR product was digested with *Sma*I and *Hin*dIII and cloned into a pGEMEX-1 vector digested with the same enzymes. The encoding region was then amplified using a T7up primer (5′-CCCGCGCGTT GGCCGATTCA-3′) and a T7term primer (5′-GCTGCGCAA CTGTTGGGAA GGGCGA-3′), from which the mRNA was transcribed by a T7 RNA polymerase as described previously.[8]

Cell-Free Translation. Each acyl–tRNA was added to an *E. coli* cell-free translation system together with the MBP mRNA containing the UAG initiator codon. The translation was performed in a reaction mixture (10 μL) containing 55 mM HEPES–KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 12 mM magnesium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphor(enol)pyruvate, 1 mM spermidine, 1.9% poly(ethylene glycol) 8000 (PEG-8000), 35 μg mL^{-1} folinic acid, 0.1 mM 20 standard amino acids, 16 μg mRNA, 90 pmol acyl–tRNA, and an *E. coli* S-30 extract (2 μL). The reaction mixture was incubated at 37 °C for 1 h. It (1 μL) was mixed with 2 × SDS-PAGE sample buffer (10 μL) and water (9 μL), and then the sample (5 μL) was subjected to 10% SDS-PAGE after boiling. Fluorescence images of the SDS-PAGE gel were measured by a fluorescence scanner (FMBIO-III, Hitachi Software Engineering) with excitation at 488 nm and emission at 520 nm. The same gel was analyzed by Western blotting using an anti-His tag antibody and an alkaline phosphatase-labeled anti-mouse IgG.

Protein Purification. The translation products were purified on Ni–nitrilotriacetic acid (NTA) magnetic beads. The translation reaction mixture (50 μL) was diluted in a HKM buffer (25 mM HEPES–KOH (pH 7.4), 100 mM KCl, and 5 mM $MgCl_2$) to a final volume of 200 μL, and mixed with MagneHis Ni-particles (20 μL). After shaking at room temperature for 30 min, the beads were washed once with the HKM buffer, once with the HKM buffer containing 8 M urea, and thrice with the HKM buffer. For MALDI-TOF MS analysis, the beads were washed once with 10 mM Tris-HCl, pH 8, and MBP was eluted with 10 mM Tris-HCl, pH 8 and 0.5 M imidazole (25 μL). For fluorescence measurements, MBP was eluted with the HKM buffer (50 μL) containing 0.5 M imidazole and 0.1% PEG-8000, and the eluate was desalted by passage through MicroSpin G-25 column equilibrated with the HKM buffer containing 0.05% Brij-35 and 0.1% PEG-8000.

MALDI-TOF MS Analysis. Purified MBP (25 μL) was mixed with 10 mM Tris-HCl, pH 8, (25 μL) and 0.01 unit μL^{-1} lysyl endopeptidase (1 μL), and incubated at 30 °C for 6 h. The resulting peptide fragments were desalted and concentrated using ZipTip C18 and eluted with a matrix solution that contained saturated α-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetonitrile and 0.1% TFA. MALDI-TOF MS analysis was performed on Voyager DE-Pro (Applied Biosystems) in a positive mode using angiotensin II as an external calibrant.

Streptavidin Binding Analysis. Purified MBP (0.5 μL) was mixed with streptavidin (final concentration 50 μM) and applied to 10% SDS-PAGE without boiling. The gel was visualized by a fluorescence scanner and Western blotting. For fluorescence spectral measurements, purified MBP (5 μL) and streptavidin (final concentration 1 μM) were diluted with the HKM buffer containing 0.005% Brij-35 and 0.1% PEG-8000 to a final volume of 200 μL in a 5 × 5 mm^2 quartz cell. Fluorescence spectra were measured from 505 to 650 nm with excitation at 490 nm on Fluorolog-3 (Horiba Jobin-Yvon) at 25 °C. Excitation and emission slit widths were set to 5 nm. Titration curves for the streptavidin binding reaction were obtained in a similar manner using a fluorescence microplate reader (Stratagene Mx3005P) with excitation at 492 nm and emission at 516 nm.

Results and Discussion

Preparation of Initiator tRNAs Acylated with Biotinylated BFLAF Derivatives. BioBFLAF, BioXBFLAF, and BioXXBFLAF were linked at the 3′ terminus of an *E. coli* initiator tRNA with a CUA anticodon by a chemical aminoacylation method.[17,18] For this purpose, aminoacylated dinucleotide pdCpA derivatives with biotinylated BFLAF derivatives were chemically synthesized by a two-step acylation, in which BODIPYFL and biotin were linked to the *p*- and α-amino groups of aminophenylalanyl–pdCpA. At first, aminophenylalanyl–pdCpA was reacted with BODIPYFL *N*-hydroxysuccinimide ester at pH 5 as described previously.[2] At pH 5, the α-amino group was fully protonated, but the *p*-amino group was partially protonated, therefore, the *p*-amino group specifically reacts with the succinimide ester. The α-amino group of the resulting BFLAF–pdCpA was reacted with biotin *N*-hydroxysuccinimide ester at a weak basic condition, in which the α-amino group retains its nucleophilicity to attack the carboxyl group of the succinimide ester. HPLC analysis for the biotinylation reaction (Supporting Information, Figure 1) indicated the biotinylation reaction proceeded nearly quantitatively. The resulting biotinylated BFLAF–pdCpA derivatives were purified on HPLC and identified by electrospray mass spectrometry. An *E. coli* initiator tRNA containing a CUA anticodon and lacking the 3′ dinucleotide was prepared by PCR of a synthetic tRNA gene followed by T7 transcription, in which a PCR primer containing two 2′-*O*-methylated ribonu-

cleotides was used to suppress an additional single base extension by T7 RNA polymerase as reported.[19] Capillary gel electrophoresis indicated that the single base extension was evidently suppressed by using the 2′-*O*-methylated PCR primer (Supporting Information, Figure 2). The biotinylated BFLAF–pdCpAs were ligated to the truncated initiator tRNA to give biotinylated BFLAF–tRNAs. HPLC analysis indicated that the aminoacylation rates were about 80% (Supporting Information, Figure 3).

Incorporation of Biotinylated BFLAF Derivatives into MBP. Incorporation of biotinylated BFLAF derivatives into the N-terminus was examined using MBP. The MBP gene was mutated to contain a TAG initiator codon immediately before Lys1 and a His tag at the C-terminus. The MBP gene was expressed in an *E. coli* cell-free translation system in the presence of the initiator tRNA with biotinylated BFLAF derivatives. The translation products were analyzed by SDS-PAGE and visualized by a fluorescent scanner. As shown in Figure 2a, fluorescent MBPs were successfully obtained for all the biotinylated BFLAF derivatives. To assess the incorporation efficiency of the biotinylated BFLAF derivatives, the fluorescence band intensities were compared, as summarized in Figure 2b. The relative band intensities for BioXBFLAF and BioXXBFLAF were 93% and 78% of that for BioBFLAF, respectively. The fact that the aminohexyl linker slightly decreases the incorporation efficiency of the biotinylated BFLAF suggests that the linker may inhibit the binding of the initiator tRNA to the ribosomal initiation complex. BFLAF without the biotin moiety was also incorporated as previously reported,[16] although the band intensity (41%) was lower than those for biotinylated BFLAF derivatives. This suggests that the acylation of the α-amino group may be required for efficient translation initiation. In addition, the acylation would prevent a decrease in the concentration of the aminoacylated tRNAs during the cell-free translation, due to the stabilization of the aminoacyl linkage between the tRNA and amino acid.

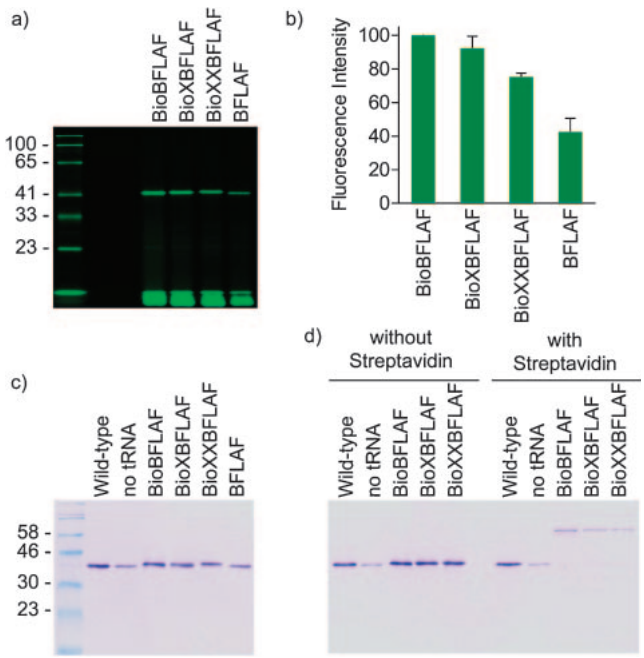

Figure 2. The incorporation of biotinylated BFLAF derivatives into the N-terminus of maltose binding protein (MBP). (a) Fluorescence image of a SDS-PAGE gel with excitation at 488 nm and emission at 520 nm. (b) Relative band intensity of the BODIPYFL-labeled MBP in the fluorescence image. The data are represented as means ± SD of four assays. (c) Western blot analysis using anti-His tag antibody. (d) Gel shift assay for the binding of MBPs containing biotinylated BFLAF derivatives with streptavidin.

The translation products were also visualized by Western blot analysis using an anti-His tag antibody (Figure 2c). A wild-type MBP, which was obtained from an mRNA with a AUG codon in place of a UAG codon, was also applied to the Western blot for comparison. The band intensities on the Western blot indicated that the BioBFLAF-containing MBP was produced in almost the same quantities as the wild-type MBP. The aminohexyl linker decreased the yield of the protein containing BioXBFLAF and BioXXBFLAF, as observed by the fluorescent SDS-PAGE analysis.

The Western blot showed that the UAG-initiating MBP mRNA produced a small amount of MBP in the absence of the initiator tRNA (Figure 2c). This may be due to the decoding of the UAG initiator codon by any endogenous aminoacyl–tRNAs. To examine whether this undesirable decoding of the UAG codon occurs even in the presence of BioBFLAF–tRNA, biotinylated, and non-biotinylated proteins were separated by a gel shift assay. The cell-free translation products were purified on Ni–NTA beads, mixed with streptavidin, and applied to Western blot analysis without heat denaturation. As shown in Figure 2d, the translation product obtained in the presence of BioBFLAF–tRNA completely shifted to the higher molecular weight region, whereas that obtained in the absence of tRNA showed no shift. This result clearly demonstrated that no non-biotinylated protein was produced in the presence of BioBFLAF–tRNA, and therefore, the UAG initiator codon was specifically decoded by BioBFLAF–tRNA. In addition, this result supported that the biotin was evidently incorporated into the protein.

The incorporation of BioBFLAF was also demonstrated by mass analysis of lysyl endopeptidase-digested peptide fragments. For this experiment, Lys1 residue was deleted from the MBP gene to avoid the digestion immediately after BioBFLAF by lysyl endopeptidase. MALDI-TOF MS analysis of the lysyl endopeptidase-digested peptide fragments showed that the expected mass was observed (calculated for BioBFLAF–IEEGK 1237.57, found 1237.50) (Figure 3), indicating that BioBFLAF was evidently incorporated into the N-terminus of MBP. A peak observed at 1218.53, which was 19 lower than the above peptide, could be identified as a peptide that lacked one fluoride possibly due to the laser ionization. A peak observed at 1189.54 could be identified as a peptide AGLTFLVDLIK derived from 190–200 residues of MBP (calculated 1189.71).

Fluorescence Properties. The fluorescence properties of the MBPs containing biotinylated BFLAF derivatives and their complex with streptavidin were investigated by fluorescence spectroscopy measurements. The concentrations of the MBPs were adjusted to be nearly equal based on the fluorescence intensities, and the initial fluorescence intensities were normalized. As shown in Figure 4, distinct fluorescence spectra

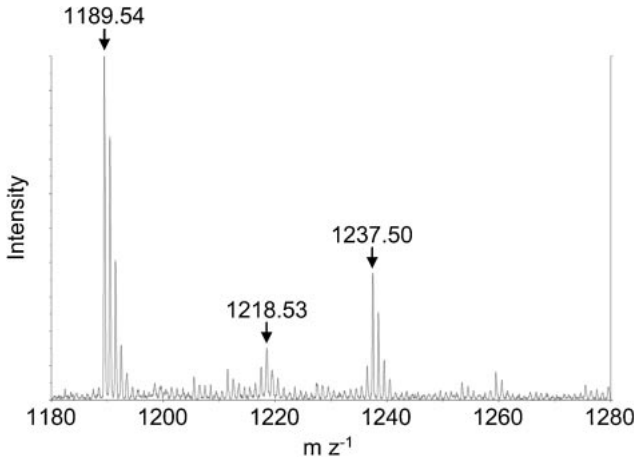

Figure 3. MALDI-TOF MS analysis of lysyl endopeptidase-digested peptide fragments of the purified MBP containing BioBFLAF at the N-terminus and lacking Lys1 residue. A peak observed at 1237.50 could be identified as a peptide BioBFLAF–IEEGK (calculated 1237.57), and a peak at 1218.53 could be identified as a peptide that lacked one fluoride possibly due to the laser ionization. A peak at 1189.54 was identified as 190–200 peptide (AGLTFLVDLIK) of MBP (calculated 1189.71).

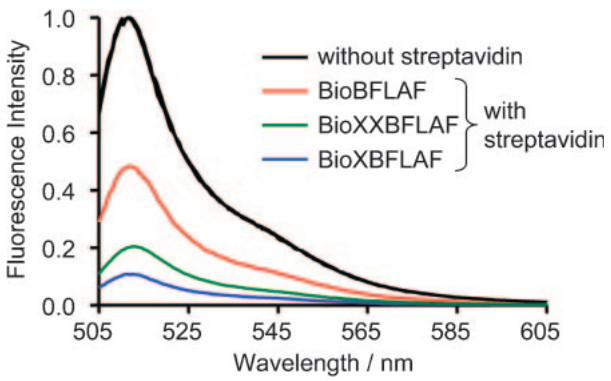

Figure 4. Fluorescence spectra of MBPs containing biotinylated BFLAF derivatives with or without streptavidin. Fluorescence intensities were normalized by those without streptavidin. The spectra of MBPs containing Bio-, BioX-, and BioXXBFLAF without streptavidin, shown in black lines, were identical to each other.

derived from BODIPYFL were observed for the purified MBPs containing biotinylated BFLAF derivatives. The spectra were completely identical to each other after normalization. The addition of streptavidin (1 μM) decreased the fluorescence intensity by 50, 90, and 80% for Bio-, BioX-, and BioXXBFLAF, respectively. The saturation of the binding of streptavidin at 1 μM was confirmed by titration curves (Supporting Information, Figure 4). It has been reported that the fluorescence of biotinylated fluorescein was significantly quenched by the binding of streptavidin.[20] The higher quenching efficiency for BioX- and BioXXBFLAF than BioBFLAF may suggest that the flexible aminohexyl linker facilitates the quenching of BODIPYFL by the binding of streptavidin. These results support the complexation of the BioBFLAF-containing proteins with streptavidin in solution and moreover, indicate

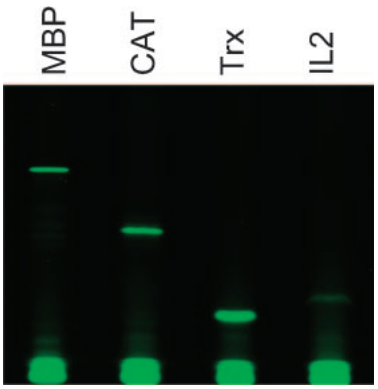

Figure 5. Fluorescence image of a SDS-PAGE gel for the incorporation of biotinylated BFLAF derivatives into the N-terminus of MBP, chroramphenicolacetyltransferase (CAT), thioredoxin (Trx), and interleukin 2 (IL2) with excitation at 488 nm and emission at 520 nm.

that the linker enhances the fluorescence quenching of BioBFLAF upon complexation with streptavidin, although it may reduce the steric hindrance upon the complexation.

Incorporation into Various Proteins. The incorporation of BioBFLAF was examined for several proteins other than MBP. As shown in Figure 5, BioBFLAF was successfully incorporated into chroramphenicolacetyltransferase (CAT), thioredoxin (Trx), and interleukin 2 (IL2) in response to the UAG initiator codon, suggesting that BioBFLAF can be incorporated into the N-terminus of proteins regardless of N-terminal amino acid sequence.

Conclusion

The present study developed a novel method for the double labeling of the N-terminus of proteins by introducing aminophenylalanine derivatives with biotin and fluorophores at the α- and *p*-amino groups. This method is a general and useful tool for N-terminal-specific modification of proteins with two molecules. The finding that the relatively large biotinylated BFLAF derivatives are efficiently accepted as substrates for the translation initiation process raises the possibility that a variety of artificial chemical groups can be introduced at the N-terminus of proteins. This combination of the four-base codon-mediated incorporation of non-natural amino acids will also be useful for the incorporation of three or more chemical groups into proteins.

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Supporting Information

Supplementary figures for HPLC analyses of biotinylated BFLAF–pdCpA derivatives and biotinylated BFLAF–tRNA, capillary gel electrophoresis of truncated tRNAs, and titration curves for the binding of MBP with streptavidin. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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