DOI: 10.1002/cbic.200800703

Position-Specific Incorporation of Fluorescent Non-natural Amino Acids into Maltose-Binding Protein for Detection of Ligand Binding by FRET and Fluorescence Quenching

Issei Iijima and Takahiro Hohsaka*[a]

Position-specific incorporation of fluorescent groups is a useful method for analysis of the functions and structures of proteins. We have developed a method for the incorporation of visible-wavelength-fluorescent non-natural amino acids into proteins in a cell-free translation system. Using this technique, we introduced one or two BODIPY-linked amino acids into maltose-binding protein (MBP) to obtain MBP derivatives showing ligand-dependent changes in fluorescence intensity or intensity ratio. BODIPY-FL-aminophenylalanine was incorporated in place of 15 tyrosines, as well as the N-terminal Lys1, and the C-terminal Lys370 of MBP. Fluorescence measurements revealed that MBP containing a BODIPY-FL moiety in place of Tyr210 showed a 13-fold increase in fluorescence upon binding of maltose. Tryptophan-to-phenylalanine substitutions suggest that the increase in fluorescence was the result of a decrease in the quenching of BODIPY-FL by tryptophan located around the binding site. MBP containing a BODIPY-558 moiety also showed a maltose-dependent increase in fluorescence. BODIPY-FL was then additionally incorporated in place of Lys1 of the BODIPY-558-containing MBP as a response to the amber codon. Fluorescence measurements with excitation of BODIPY-FL showed a large change in fluorescence intensity ratio (0.13 to 1.25) upon binding of maltose; this change can be attributed to fluorescence resonance energy transfer (FRET) and maltose-dependent quenching of BODIPY-558. These results demonstrate the usefulness of the position-specific incorporation of fluorescent amino acids in the fluorescence-based detection of protein functions.

Introduction

Incorporation of fluorescent groups into proteins has been used to analyze structures, functions, localizations, and structural changes of proteins. The incorporation is usually done by a chemical modification, although it is not easy to introduce fluorescent groups into proteins in a position-specific and quantitative fashion. Thiol-specific chemical modification after the replacement of amino acids at desired positions by cysteine may be used to attach fluorescent groups at specific positions. This strategy, however, does not always result in quantitative chemical reactions and, in addition, cysteine replacement may affect the structure and function of a protein. Fusion expression with green fluorescent protein (GFP) is restricted to N- or C-terminal labeling, and the large GFP molecule is likely to cause steric hindrance. Several semisynthetic approaches, such as native chemical ligation and protein trans-splicing have been used for the position-specific introduction of fluorescent groups.[1–3]

Alternatively, position-specific and quantitative incorporation of small fluorescent groups has also been achieved by introducing fluorescent non-natural amino acids into proteins as a response either to an amber codon UAG or to four-base codons in cell-free or in vivo translation systems.[4–9] Recently, we have developed the green and red fluorescent non-natural amino acids BODIPY-FL- and BODIPY-558-linked *p*-amino-L-phenylalanine (BFLAF and B558AF; Figure 1), designed to be accepted as substrates for translational machinery.[10] These two fluorescent amino acids were used for the analysis of structural changes in calmodulin by fluorescence resonance energy transfer (FRET). The use of highly fluorescent BODIPY-linked amino acids might compensate for the low yields of non-natural amino acid-containing proteins. These low yields result from low productivity of the cell-free translation systems, competitive triplet decoding of the four-base codons, or translation termination at the amber codon.

The fluorescence of BODIPY groups has been reported to be influenced by microenvironmental changes around them[11] and, in particular cases, by tryptophan quenching.[12,13] The incorporation of fluorescent BODIPY groups into appropriate positions in proteins should enable us to detect protein structural changes as fluorescence intensity changes, as demonstrated for fluorescently labeled peptides.[14] Although chemical modification is not suitable for the introduction of fluorescent groups at specific positions, the incorporation of BODIPY amino acids in response to expanded codons overcomes this problem and, in addition, allows easy screening of incorporation positions.

Here, we introduced one or two BODIPY-linked fluorescent non-natural amino acids into maltose-binding protein (MBP) to

[a] *I. Iijima, Prof. T. Hohsaka*
School of Materials Science
Japan Advanced Institute of Science and Technology
1-1 Asahidai, Nomi, Ishikawa 923-1292 (Japan)
Fax: (+81) 761-51-1149
E-mail: hohsaka@jaist.ac.jp

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200800703.

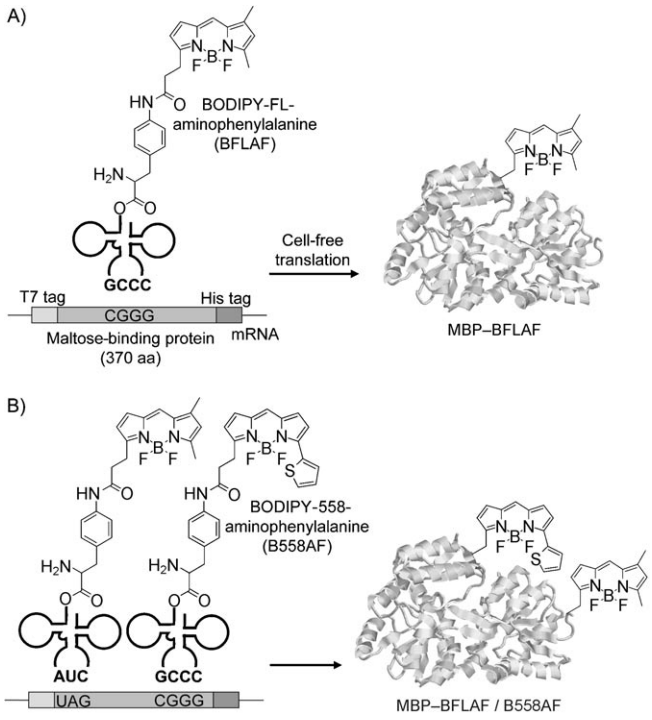

Figure 1. A) Schematic illustration of the incorporation of BODIPY-FL-aminophenylalanine into MBP in response to the CGGG codon, and B) the double incorporation of BODIPY-FL- and BODIPY558-aminophenylalanines in response to the CGGG and UAG codons.

obtain MBP derivatives with ligand-dependent changes in fluorescence intensity or intensity ratio. MBP is an important protein in maltose transport in *Escherichia coli*, and its conformational change on substrate binding has been well characterized by X-ray crystallographic analysis.[15,16] In addition, several studies have reported fluorescence sensing of the substrate binding of MBP as a result of chemical modification or fluorescent protein fusion.[17,18]

In the first step, we introduced BFLAF into all 15 tyrosine positions in MBP, as well as its N-terminal Lys1 and C-terminal Lys370 positions, as a response to the four-base codon CGGG in an *E. coli* cell-free translation system (Figure 1A). The MBPs were analyzed for changes in fluorescence on addition of maltose, in order to identify the incorporation position corresponding to the observation of a large change in fluorescence. In the second step, BFLAF and B558AF were incorporated at Lys1 and at the identified position, respectively (Figure 1B), for the fluorescence ratio detection of maltose by FRET and fluorescence quenching.

Results

Position-specific incorporation of BODIPY-FL into MBP

BFLAF was incorporated into the 15 tyrosine positions in MBP, as well as the N-terminal Lys1 position and the C-terminal Lys370 position. Tyrosine residues, each containing a hydrophobic benzene ring and a hydrophilic hydroxy group, are frequently located on the protein surface. The three-dimensional structure of MBP (Figure 2A) indicates that the tyrosine residues other than Tyr70, Tyr106, and Tyr155 are located on the protein surface. Upon introduction of BFLAF into these tyrosine positions, the phenyl ring of BFLAF would be expected to occupy the hydrophobic space originally occupied by the phenyl ring of tyrosine. Also, the BODIPY-FL at the *p*-position would be located on the protein's surface, at the position where the hydroxyl group of tyrosine was originally located. Therefore, it would be expected that BODIPY-FL should not seriously affect the protein structure and function, but would change its location on the protein surface when local structural change occurs around the tyrosine position. The incorporation

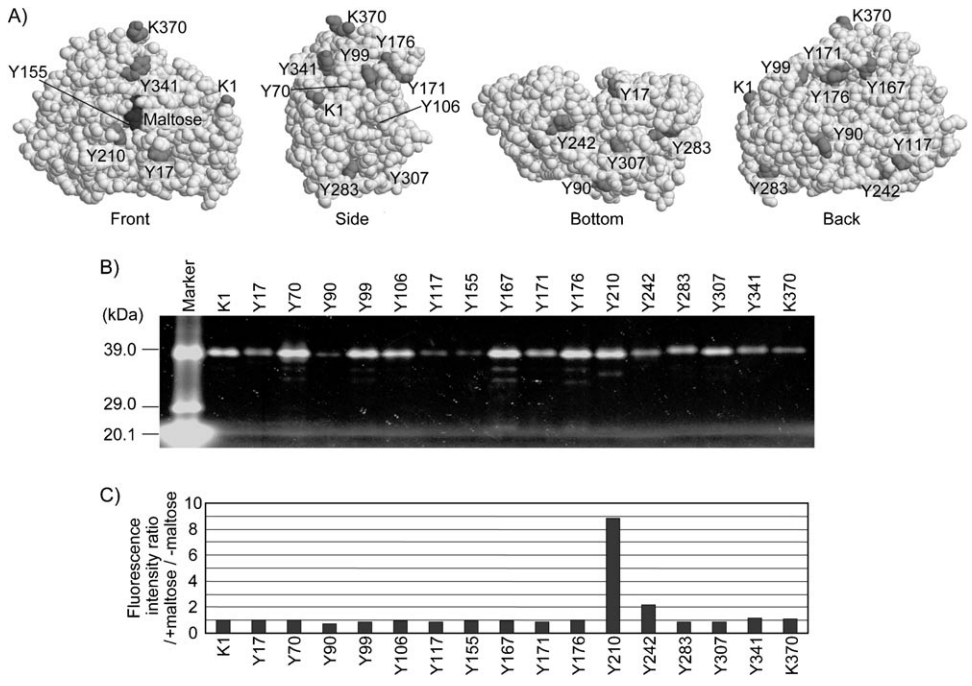

Figure 2. A) Three-dimensional structure of MBP with maltose (PDB code 1ANF). Tyrosine residues, Lys1, and Lys370 are colored gray, and maltose is colored black. B) Fluorescence image of a SDS-PAGE gel of purified MBPs containing BFLAF at their 15 tyrosine positions and at Lys1 and Lys370, with excitation at 488 nm and emission at 520 nm. The fluorescent band at the position of the front of electrophoresis (at 20 kDa) was observed for BPB-containing sample buffer only, and was not therefore derived from translation products. C) Fluorescence intensity ratios of BFLAF-containing MBPs with and without maltose (4×10^{-4} M) at maximum emission wavelength with excitation at 490 nm.

of BFLAF at the N or C termini would also be expected to have little influence on the structure and function of MBP.

Incorporation of BFLAF was carried out by a four-base codon method. The four-base codon CGGG was introduced into each of the positions of the MBP gene, which contained a T7 tag and a His tag at the N and C termini, respectively. A yeast phenylalanine tRNA containing a CCCG four-base anticodon and aminoacylated with BFLAF was synthesized by the chemical aminoacylation method as described previously.[10] The BFLAF-containing MBPs, such as Y17BFLAF, were synthesized in an *E. coli* cell-free translation system.

The fluorescence images of the SDS-PAGE gel for unpurified (Figure S1 in the Supporting Information) and His tag-purified translation products (Figure 2B) showed that fluorescent bands were observed at the expected molecular weight (40 kDa) for all the incorporation positions; this suggests the successful incorporation of BFLAF. The intensities of the fluorescent bands for the unpurified products were different, depending on the incorporation position. Incorporation at Tyr17, Tyr90, Tyr117, and Tyr155 was less efficient, even though sufficient fluorescent MBPs for the subsequent fluorescence measurements were obtained. The translation products were also analyzed by Western blotting with use of an anti-T7 tag antibody. Bands with the same mobility as the wild-type MBP were observed for all positions (Figure S1). As for the fluorescence image, the Tyr17, Tyr90, Tyr117, and Tyr155 positions produced very faint bands. Truncated proteins were observed for positions Tyr117–341, which correspond to the translation products generated when CGGG is decoded as a triplet and the downstream stop codon on the +1 frame terminates peptide elongation. The short truncated proteins for positions Lys1 to Tyr106 should have been generated but would not have been observed under these SDS-PAGE conditions because of their low molecular weights.

Evaluation of the maltose-binding activities of MBP derivatives that contain BFLAF moieties

The maltose-binding activities of the purified MBP derivatives were evaluated by use of amylose-coated magnetic beads. If the MBP were to maintain binding activity for maltose, it would be immobilized on the amylose-coated beads and would not be present in the supernatant. In the co-presence of maltose, however, the MBP would not be immobilized but would remain in the supernatant as a complex with maltose. The maltose-binding activity of an MBP derivative can therefore be evaluated by analyzing the supernatants of the amylose beads in the presence or absence of maltose. The SDS-PAGE results (Figure S2) show that, for all incorporation positions, fluorescent bands were detected in the supernatants in the presence of maltose but not in its absence. This result supports the idea that the BFLAF-containing MBP derivatives did not lose their maltose-binding activities.

Fluorescence analysis of MBP derivatives upon titration with maltose

The fluorescence spectra of BFLAF-containing MBP derivatives were measured, and the ratios of fluorescence intensities in the absence and in the presence of maltose (4×10^{-4} M) are summarized in Figure 2C. The relative fluorescence intensities of the BFLAF-containing MBP derivatives were estimated as summarized in Table S1 by correction of the fluorescence intensities with the relative concentrations of the purified MBPs as determined from the fluorescence band intensities on the SDS-PAGE. For most positions the fluorescence intensities remained unchanged upon addition of maltose, but in the cases of Tyr210 and Tyr242, nine- and twofold increases in fluorescence intensity were observed at 4×10^{-4} M of maltose. The fluorescence spectra of Y210BFLAF upon titration with maltose (Figure 3A) showed a 13-fold increase in fluorescence intensity at 1×10^{-2} M of maltose. By curve-fitting of the fluorescence intensities (Figure 3B), the K_d value of Y210BFLAF to maltose was estimated to be 5.9×10^{-5} M. The maltose-binding activity of Y210BFLAF was thus somewhat depressed relative to that of the wild-type MBP, which has a K_d value of 1×10^{-6} M.[19] The K_d value of Y242BFLAF was estimated from the increase in fluorescence intensity (Figure S3) to be 1.0×10^{-6} M; this indicates that the maltose-binding activity was not affected by the incorporation of BODIPY-FL at the Tyr242 position.

The increases in fluorescence intensity upon addition of maltose suggest that in the absence of maltose the fluorescence of BODIPY-FL incorporated at the Tyr210 and Tyr242 positions is quenched by certain amino acid residues (probably tryptophan residues), and that this quenching is depressed by the binding of maltose. The relative fluorescence intensities of the BFLAF-containing MBP derivatives (Table S1) suggest that the quenching also occurs when the BODIPY-FL is incorporated at positions other than Tyr210 or Tyr242, but is not enhanced or reduced upon the binding of maltose. Urea-induced denaturation increased the fluorescence intensity of Y210BFLAF and Y242BFLAF (Figure S4); this supports the idea that BODIPY-FL

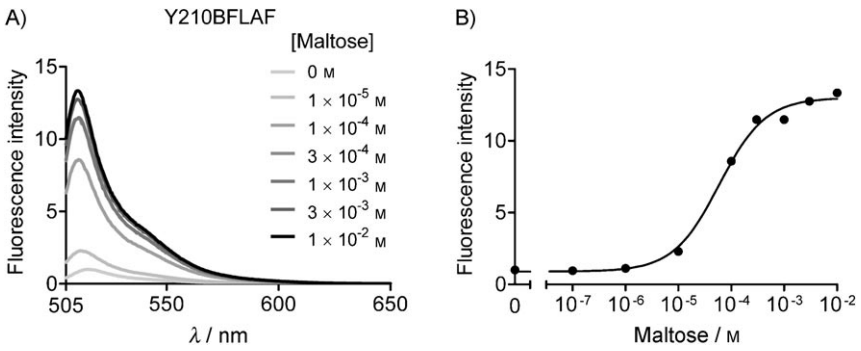

Figure 3. A) Fluorescence spectra, and B) titration curve of Y210BFLAF with excitation at 490 nm in the absence and presence of maltose. Fluorescence intensities are relative values with respect to those in the absence of maltose.

at Tyr210 and Tyr242 is quenched by neighboring residues on the native conformation.

In order to identify the amino acid residues that quench BODIPY-FL fluorescence, tryptophan residues Trp62, Trp230, Trp232, and Trp340, which are located near Tyr210 and Tyr242 (Figure 4A), were replaced with phenylalanine. Fluorescence

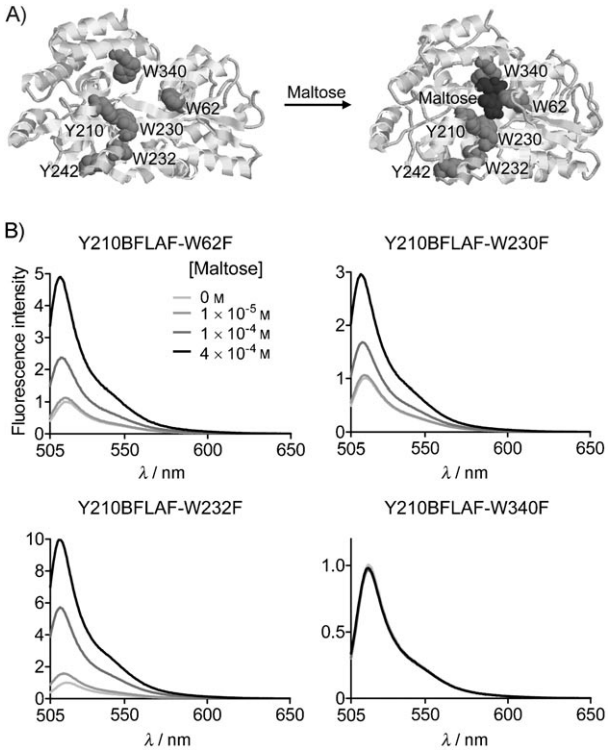

Figure 4. A) Three-dimensional structure of MBP without maltose (PDB code 1OMP) and with maltose (PDB code 1ANF). Tyr210, Tyr242, and tryptophan residues located around the tyrosine residues are colored gray. B) Fluorescence spectra of Y210BFLAF containing the W62F, W230F, W232F, or W340F mutations with excitation at 490 nm in the absence and in the presence of maltose.

images of the SDS-PAGE gel and amylose bead assay showed that BFLAF was successfully incorporated into these tryptophan-substituted MBPs and that the resulting MBPs retained maltose-binding activity (Figure S5). Fluorescence measurements revealed that the W340F mutant showed no increase in fluorescence intensity upon titration with maltose, whereas W62F, W230F, and W232F mutants showed significant increases (Figure 4B). For Y242BFLAF, only the W232F mutant showed no increase in fluorescence intensity (Figure S6). These results indicate that BODIPY-FL at Tyr210 and Tyr242 is predominantly quenched by Trp340 and Trp232, respectively.

Fluorescence analysis of MBP derivatives that contain BODIPY-558 moieties

We have previously shown that both B558AF and BFLAF can be incorporated into proteins.[10] To examine the possibility of fluorescence changes for another BODIPY fluorophore, B558AF was incorporated into Tyr210 and Tyr242 of MBP. Fluorescence images of the SDS-PAGE gel and amylose bead assays showed that B558AF had been successfully incorporated and that the resulting MBPs retained their maltose-binding activity (Figure S7). Fluorescence measurements on titration with maltose indicated that the fluorescence of B558AF increased 11- and 1.8-fold for Y210B558AF and Y242B558AF, respectively (Figures 5 and S8), as in the case of BFLAF. From fitting of the fluorescence intensity changes, the K_d values of Y210B558AF and Y242B558AF for maltose were estimated to be 4.0×10^{-4} and 5.2×10^{-6} M, respectively, which were comparable to those of Y210BFLAF and Y242BFLAF.

Fluorescence quenching and FRET analysis of MBP derivatives that contain two fluorescent amino acids

Maltose binding can be detected in the form of increases in the fluorescence intensities of MBP derivatives that contain BODIPY moieties at the Tyr210 or Tyr242 positions. However, quantitative detection of maltose is not achieved unless a dose-response curve is obtained at the defined concentration of the MBP. On the other hand, we have reported that two BODIPY-linked amino acids can be incorporated into two different positions of calmodulin by use of two four-base codons.[10] The resulting doubly labeled calmodulin showed that the FRET change depended on the conformational change upon the binding of calmodulin-binding peptide. The change in fluorescence intensity ratio determined from the FRET change allowed quantitative detection of the calmodulin-binding peptide.

In this study, quantitative detection of maltose binding without determination of the concentration of MBP was investigated by introducing BFLAF in place of Lys1 and B558AF in place of Tyr210. The C^{α}–C^{α} distance from Lys1 to Tyr210 is 43 Å, from the crystallographic data (PDB, 1OMP).[17] Because this value is smaller than the Förster distance for BODIPY-FL and BODIPY-558 (calculated to be 59.6 Å on the assumption that the orien-

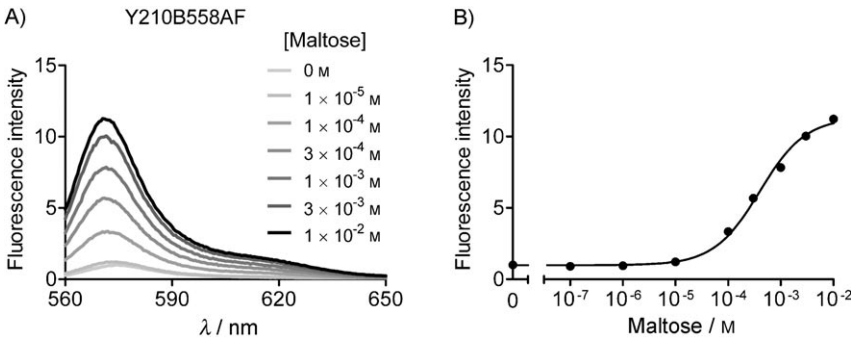

Figure 5. A) Fluorescence spectra, and B) titration curve of Y210B558AF with excitation at 545 nm in the absence and presence of maltose.

tation factor (κ^2) is 2/3) FRET would be expected to occur between BFLAF at Lys1 and B558AF at Tyr210. In the absence of maltose, however, BODIPY-558 at Tyr210 should be quenched after accepting the excitation energy from BODIPY-FL by FRET, and fluorescence of BODIPY-FL should therefore be predominantly observed, as illustrated in Figure 6A. On the other hand, in the presence of maltose, fluorescence quenching should be depressed and fluorescence of both BODIPY-FL and BODIPY-558 should be observed. Measurement of the fluorescence intensity ratio of BODIPY-FL and BODIPY-558 should allow quantitative detection of maltose.

To incorporate the second fluorescent amino acid, the amber codon TAG was additionally introduced into Lys1 of the MBP gene that contained the CGGG codon at Tyr210. The amber codon was decoded by a highly efficient amber suppressor tRNA derived from *Mycoplasma capricolum* Trp_1 tRNA,[20] which was aminoacylated with BFLAF. Fluorescence imaging of the SDS-PAGE gel indicated that MBP derivative containing two fluorescent amino acids and showing fluorescence of both BODIPY-FL and BODIPY-558 was successfully synthesized (Figure S9). The maltose-binding activity of the purified MBP was confirmed by amylose bead assay.

Fluorescence spectra showed that the fluorescence of BODIPY-558 greatly increased and the fluorescence of BODIPY-FL significantly decreased upon titration of maltose with excitation at 490 nm (Figure 6B). The decrease in BODIPY-FL fluorescence is not consistent with the result for K1BFLAF and might be the result of the increase in FRET efficiency, because the C^{α}–C^{α} distance between Lys1 and Tyr210 decreases from 43 Å to 35 Å upon binding of maltose (from crystallographic data; PDB ID: 1OMP and 1ANF). While the FRET change might increase the fluorescence intensity of BODIPY-558 to some extent, most of the increase should result from the decrease in tryptophan quenching. The fluorescence intensity ratio at 511 nm and 572 nm increased from 0.13 to 1.25 depending on the maltose concentration (Figure 6C). By fitting of the ratio curve, the K_d value for maltose was estimated to be 5.1×10^{-4} M, which was nearly the same as that of Y210B558AF.

In the case of K1BFLAF-Y242B558AF, the fluorescence of BODIPY558 slightly increased, while that of BODIPY-FL remained nearly constant (Figure S10). The fluorescence intensity ratio curve indicated that the K_d value was 6.3×10^{-6} M, which was also nearly the same as that of Y242B558AF.

The change in the fluorescence intensity ratio for K1BFLAF-Y210B558AF was visualized on a microplate at maltose concentrations from 1×10^{-5} to 1×10^{-2} M. The fluorescence change from green to yellow with excitation at 488 nm that would indicate that MBP containing two fluorescent amino acids has potential for quantitative imaging of maltose binding, was successfully observed (Figure 6D).

Discussion

The fluorescence images of the SDS-PAGE gel indicate successful incorporation of BFLAF at the Lys1, Lys370, and all 15 tyrosine positions in MBP. Quantification of the fluorescent bands, however, indicated that the incorporation efficiency of BFLAF depended on its incorporation position. On the other hand, *p*-aminophenylalanine, without a fluorophore, has been efficiently incorporated at all positions in MBP.[21] These results suggest that the incorporation of non-natural amino acids with rather bulky side chains, such as BODIPY-FL, is affected by sequence context, although those possessing side chains similar to those of the natural amino acids can be efficiently incorporated at any positions. Sequence context such as secondary structure of mRNA may enhance the triplet decoding of the four-base codon and reduce the incorporation of non-natural amino acids that are less efficiently accepted as substrates in ribosome.

The amylose bead assay showed that all BFLAF-containing MBP derivatives maintained maltose-binding activity. This suggests that BFLAF does not

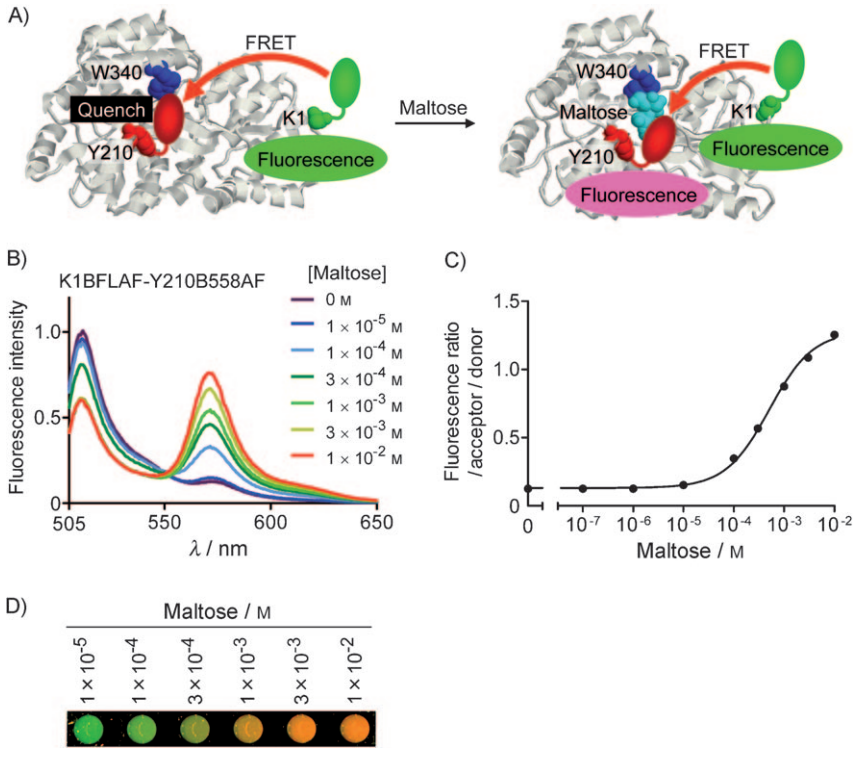

Figure 6. A) Illustration of FRET and maltose-dependent fluorescence quenching for K1BFLAF-Y210B558AF. B) Fluorescence spectra of K1BFLAF-Y210B558AF with excitation at 490 nm in the absence and in the presence of maltose. C) Titration curve of fluorescence intensity ratio at 511 nm and 572 nm. D) Fluorescence ratio imaging of K1BFLAF-Y210B558AF on a microplate in the presence of maltose with excitation at 488 nm and emission at 520 nm (green) and 605 nm (red).

have a critical influence on the binding activity of MBP; this is possibly because BFLAF is not too bulky to be a steric hindrance. According to the three-dimensional structure, the *p*-hydroxy groups of Tyr70, Tyr106, and Tyr155 are buried (Figure 2A). The fact that BFLAF incorporation at these positions did not abolish the binding activity suggests that MBP can flexibly change its structure to reduce the steric hindrance of BODIPY-FL.

Fluorescence measurements with the BFLAF-containing MBP derivatives revealed that BFLAF fluorescence increased markedly upon binding of maltose when incorporated in place of Tyr210 or Tyr242. The fluorescence change upon urea-induced denaturation supports the idea that BODIPY-FL at these tyrosine residues is quenched by neighboring residues in the native conformation. The midpoints of denaturation transition were 2.65 and 2.61 M for Y210BFLAF and Y242BFLAF, respectively. Comparison with the value for wild-type MBP (3.5 M)[22] suggests that the incorporation of BFLAF may partially destabilize the conformation of MBP.

We speculated that fluorescence quenching is caused by tryptophan residues located around the Tyr210 or Tyr242 residues. The three-dimensional structure of maltose-free MBP (Figure 4A) shows that Tyr210 is located in proximity to Trp62, Trp230, Trp232, and Trp340, which form the maltose-binding cavity. In the maltose-bound form, on the other hand, these tryptophan residues change their locations to make contacts with maltose.[16] Similarly, Tyr242 is located in proximity to Trp230 and Trp232 in the maltose-free form. The fluorescence measurements for the tryptophan-substituted mutants indicate that BODIPY-FL at Y210 and at Y242 is predominantly quenched by Trp340 and Trp232, respectively. The intense quenching and the large K_d value for Y210BFLAF suggest that BODIPY-FL at Tyr210 may strongly interact with Trp340 and prevent the interaction between Trp340 and maltose. On the other hand, the K_d value for Y242BFLAF is nearly the same as that of wild-type MBP and fluorescence quenching is less efficient; this suggests that BODIPY-FL at Tyr242 may interact weakly with Trp232.

Although the intensity change on substrate binding produced by the single fluorophore was successfully observed, the incorporation of a second fluorophore is effective for the quantitative detection of the substrate. Some saccharide-binding proteins that show substrate-dependent fluorescence ratio changes have been synthesized by a chemical modification technique.[23,24] In the present study, BFLAF and B558AF were incorporated in place of Lys1 and either Tyr210 or Tyr242, respectively, to enable the detection of maltose binding by monitoring of the fluorescence intensity ratio of the two fluorophores. The fluorescence of BODIPY558 with excitation at 490 nm means that FRET from BODIPY-FL to BODIPY558 occurs. The increase in the fluorescence intensity of BODIPY558 on addition of maltose can be explained as a result of the decrease in tryptophan quenching that is observed in Y210B558AF. The fluorescence intensity of BODIPY-FL significantly decreased on addition of maltose, indicating the increase in FRET efficiency. The decrease in tryptophan quenching and the increase in FRET efficiency contribute to the marked change in the fluorescence intensity ratio, and this enables sensitive detection of maltose.

The incorporation of two bulky fluorophores may decrease protein function, but incorporation of a second fluorescent amino acid at the N terminus is an effective way of preventing such loss of protein function. The ratio curves for Y210B558AF and K1BFLAF-Y210B558AF were nearly identical; this suggests that maltose-binding activity is not seriously affected by the incorporation of BFLAF at N-terminal Lys1.

The fluorescence ratio image of K1BFLAF-Y210B558AF in the presence of various concentrations of maltose on a microplate suggests the possibility of quantitative imaging of maltose binding. It has been reported that MBP fused with cyan and yellow fluorescent proteins (CFP and YFP, respectively) shows potential for quantitative detection of maltose through measurement of the fluorescence intensity ratio.[25] However, the ratio change is much smaller than that seen with K1BFLAF-Y210B558AF, possibly because CFP and YFP at the N and C termini are inappropriate for the FRET change. The unlimited possibilities for incorporation position, in addition to the much smaller size of the fluorophores, are an advantage of this method over the fluorescent protein fusion method. Transfection of fluorescent amino acid-containing proteins into living cells should enable the fluorescence ratio imaging of various ligands.

This strategy would be expected to allow analysis of various proteins through the introduction of the BODIPY fluorescent amino acid in the vicinity of tryptophan. Additional incorporation of another fluorescent amino acid provides a useful and general method for quantitative detection and imaging of ligand–protein interactions through FRET and ligand-dependent fluorescence quenching. This study demonstrates that the position-specific incorporation of a fluorescent amino acid is a useful strategy by which to determine appropriate incorporation positions for ligand-dependent fluorescence quenching.

Experimental Section

Materials: pMAL-2X vector was purchased from New England Biolabs (Ipswich, MA, USA). *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). The MicroSpin G25 column was from GE Healthcare Bio-Science (Piscataway, NJ, USA). The MagExtractor-MBP (amylose-coated magnetic bead kit was from Toyobo (Osaka, Japan). T4 RNA ligase was from Takara Bio (Otsu, Japan). BODIPY-FL and BODIPY558 succinimide esters were from Invitrogen (Carlsbad, CA, USA). Maltose monohydrate was from Nacalai Tesque (Kyoto, Japan). Urea ultrapure grade was from MP Biomedicals (Solon, OH, USA).

Preparation of MBP mRNAs: An MBP gene was obtained from the pMAL-p2X vector and the coding region was cloned into the EcoRI and HindIII sites of the pGSH vector[4] to attach the T7 and hexahistidine tags at the N and C termini, respectively. The EcoRI sequence was then deleted to eliminate the addition of amino acid residues between the T7 tag and MBP.

The four-base codon CGGG was introduced into the 15 tyrosine positions (17, 70, 90, 99, 106, 117, 155, 167, 171, 176, 210, 242, 283, 307, and 341), the N-terminal Lys1 position, and the C-terminal Lys370 position of the MBP gene, as described previously.[21] To incorporate the second fluorescent amino acid, an amber codon (TAG) was additionally introduced into the Lys1 position of the CGGG-containing MBP genes. For substitution of tryptophan residues with phenylalanine, TGG codons for W62, W230, W232, and W340 were each replaced by TTC.

The encoding region of the MBP genes was amplified by PCR with use of the T7up primer (CCC GCG CGT TGG CCG ATT CA) and the T7term primer (TAT TAC GCC AGG TTA TCC GG), and the resulting DNA was transcribed with T7 RNA polymerase as described previously.[4]

Preparation of aminoacyl tRNAs: A yeast phenylalanine tRNA, which contained the four-base anticodon CCCG and lacked two nucleotides at its 3′-end, was prepared by PCR and T7 transcription as described previously with a 3′ primer [G(2′-*O*-Me)-U(2′-*O*-Me)-GCG AAT TCT GTG GAT CGA] containing two 2′-*O*-methylated ribonucleotides at the 5′ end.[26] An amber suppressor tRNA derived from *Mycoplasma capricolum* Trp_1 tRNA[20] was prepared in a similar manner.

Ligation of the truncated tRNA and pdCpA aminoacylated with BFLAF[10] was carried out in a reaction mixture (50 μL) containing truncated tRNA (0.85 nmol), BFLAF-pdCpA (11 nmol), HEPES-Na (55 mM, pH 7.5), ATP (1 mM), $MgCl_2$ (15 mM), DTT (3.3 mM), BSA (20 $\mu g\,mL^{-1}$), and T4 RNA ligase (90 units). After incubation at 4 °C for 2 h, potassium acetate (pH 4.5) was added to the reaction mixture to obtain a final concentration of 0.3 M. BFLAF-tRNA was isolated by extraction with phenol/chloroform (1:1, *v/v*) and chloroform, and then by ethanol precipitation. The precipitate was dissolved in prechilled potassium acetate (1 mM, pH 4.5) just before addition to a cell-free translation system. Typical yield of the aminoacylated tRNA was 0.27 nmol per 50 μL of ligation reaction. B558AF-tRNA containing the four-base anticodon CCCG and BFLAF-$tRNA_{CUA}$ containing the CUA anticodon were synthesized in a similar manner.

Cell-free translation and purification of MBP derivatives containing fluorescent amino acids: Cell-free translation was carried out in a reaction mixture (50 μL) containing HEPES/KOH (55 mM, pH 7.5), GluK (210 mM), CH_3COONH_4 (6.9 mM), $(CH_3COO)_2Mg$ (12 mM), DTT (1.7 mM), ATP (1.2 mM), GTP (0.28 mM), phosphoenolpyruvate (26 mM), spermidine (1 mM), PEG-8000 (1.9%), folinic acid (35 $\mu g\,mL^{-1}$), the 19 standard proteinogenic amino acids other than arginine (0.1 mM of each), arginine (0.01 mM), mRNA (40 μg), aminoacylated tRNA (0.27 nmol), and *E. coli* S30 extract (10 μL). The reaction mixture was incubated at 37 °C for 1 h. To obtain the MBP derivatives containing two fluorescent amino acids, B558AF-tRNA and BFLAF-$tRNA_{CUA}$ were added to the cell-free translation system together with an MBP mRNA containing UAG at Lys1 and CGGG at Tyr210 or Tyr242.

After the translation reaction, the reaction mixture (0.25 μL for MBP derivatives with one fluorescent amino acid, or 0.5 μL for MBP derivatives with two fluorescent amino acids) was separated by SDS-PAGE (10%) and the gel was analyzed with a fluorescence scanner (FMBIO-III; Hitachi Software Engineering). The gel was further analyzed by Western blotting with an anti-T7 antibody (1:10000) and an alkaline phosphate-labeled anti-mouse IgG antibody (1:5000).

The synthesized MBP was purified on Ni-NTA coated magnetic beads. The translation reaction mixture (50 μL) was diluted in HKM buffer [HEPES/KOH (25 mM, pH 7.4), KCl (100 mM), $MgCl_2$ (5 mM)] 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 HKM buffer, once with HKM buffer containing urea (8 M), and thrice with HKM buffer. The MBP was eluted with HKM buffer (50 μL) containing imidazole (500 mM) and PEG-8000 (0.1%), and the eluate was desalted by passage through a MicroSpin G25 column equilibrated with HKM buffer containing Brij-35 (0.05%) and PEG-8000 (0.1%). The purity of the fluorescent MBP was confirmed by applying the purified solution (1 μL) to SDS-PAGE (10%).

Evaluation of maltose-binding activity by use of amylose-coated beads: The purified MBP derivative (2 μL) was diluted with HKM buffer or a maltose-containing buffer supplied with a kit to a final volume of 20 μL. Amylose-coated beads (2 μL) were added to this solution, and the resulting mixture was shaken at room temperature for 2 min. The supernatant was subjected to SDS-PAGE (10%) and analyzed with a fluorescence scanner. The binding activity of the MBP derivative was confirmed by the finding that the MBP derivative was present in the supernatant only when maltose-containing buffer was used as a dilution buffer.

Fluorescence measurements of MBP derivatives: The purified MBP derivative (10 μL) was diluted with HKM buffer containing Brij-35 (0.005%) and PEG-8000 (0.1%) to a final volume of 200 μL in a 5×5 mm quartz cell. Fluorescence spectra were measured from 505 to 650 nm with excitation at 490 nm for BODIPY-FL-containing MBP derivatives on a Fluorolog-3 instrument (Horiba Jobin–Yvon) at 25 °C. Excitation and emission slit widths were set to 5.0 nm. For MBP derivatives containing BODIPY558 units, fluorescence spectra were measured from 560 nm to 650 nm with excitation at 545 nm. For MBP derivatives containing BFLAF and B558AF, fluorescence spectra were measured from 505 nm to 650 nm with excitation at 490 nm, and the fluorescence intensity ratio was calculated as I_A/I_D, where I_A and I_D are the fluorescence intensities at 572 nm and 511 nm, respectively. The dissociation constant (K_d) values were calculated by curve fitting of the fluorescence intensities at maximum emission wavelength or the fluorescence intensity ratios (I_A/I_D) with use of a sigmoidal dose-response model based on Graphpad Prism (Graphpad, San Diego, CA, USA).

For urea-induced denaturation, urea was added to a purified MBP derivative to produce a final concentration of 0–8 M, and the resulting solution was incubated at 30 °C for 30 min prior to the fluorescence spectrum measurements.

For fluorescence ratio imaging of the maltose-binding activity of the MBP derivative, the purified MBP derivative containing BFLAF and B558AF (20 μL) was diluted with HKM buffer containing Brij-35 (0.005%), PEG-8000 (0.1%), and maltose to a final volume of 200 μL in a 96-well microplate. The plate was visualized with a fluorescence scanner with excitation at 488 nm and emission at 520 nm and 605 nm.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (B) (19370063) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. I.I. received a Research Fellowship for Young Scientists from the Japan Society for Promotion of Science.

Keywords: fluorescence quenching · fluorescent probes · four-base codons · FRET · protein modifications

[1] B. Schuler, L. K. Pannell, *Bioconjugate Chem.* **2002**, *13*, 1039–1043.
[2] C. Ludwig, M. Pfeiff, U. Linne, H. D. Mootz, *Angew. Chem.* **2006**, *118*, 5343–5347; *Angew. Chem. Int. Ed.* **2006**, *45*, 5218–5221.
[3] V. Muralidharan, T. W. Muir, *Nat. Methods* **2006**, *3*, 429–438.
[4] T. Hohsaka, D. Kajihara, Y. Ashizuka, H. Murakami, M. Sisido, *J. Am. Chem. Soc.* **1999**, *121*, 34–40.
[5] T. Hohsaka, N. Muranaka, C. Komiyama, K. Matsui, S. Takaura, R. Abe, H. Murakami, M. Sisido, *FEBS Lett.* **2004**, *560*, 173–177.
[6] V. W. Cornish, D. R. Benson, C. A. Altenbach, K. Hideg, W. L. Hubbell, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2910–2914.
[7] G. Turcatti, K. Nemeth, M. D. Edgerton, U. Meseth, F. Talabot, M. Peitsch, J. Knowles, H. Vogel, A. Chollet, *J. Biol. Chem.* **1996**, *271*, 19991–19998.
[8] B. E. Cohen, T. B. McAnaney, E. S. Park, Y. N. Jan, S. G. Boxer, L. Y. Jan, *Science* **2002**, *296*, 1700–1703.
[9] R. D. Anderson, III, J. Zhou, S. M. Hecht, *J. Am. Chem. Soc.* **2002**, *124*, 9674–9675.
[10] D. Kajihara, R. Abe, I. Iijima, C. Komiyama, M. Sisido, T. Hohsaka, *Nat. Methods* **2006**, *3*, 923–929.
[11] J. Karolin, L. B.-A Johansson, L. Strandberg, T. Ny, *J. Am. Chem. Soc.* **1994**, *116*, 7801–7806.
[12] N. Marmé, J.-P. Knemeyer, M. Sauer, J. Wolfrum, *Bioconjugate Chem.* **2003**, *14*, 1133–1139.
[13] S. Doose, H. Neuweiler, M. Sauer, *ChemPhysChem* **2005**, *6*, 2277–2285.
[14] H. Neuweiler, A. Schulz, A. C. Vaiana, J. C. Smith, S. Kaul, J. Wolfrum, M. Sauer, *Angew. Chem.* **2002**, *114*, 4964–4968; *Angew. Chem. Int. Ed.* **2002**, *41*, 4769–4773.
[15] A. J. Sharff, L. E. Rodseth, J. C. Spurlino, F. A. Quiocho, *Biochemistry* **1992**, *31*, 10657–10663.
[16] F. A. Quiocho, J. C. Spurlino, L. E. Rodseth, *Structure* **1997**, *5*, 997–1015.
[17] G. Gilardi, L. Q. Zhou, L. Hibbert, A. E. G. Cass, *Anal. Chem.* **1994**, *66*, 3840–3847.
[18] I. L. Medintz, J. R. Deschamps, *Curr. Opin. Biotechnol.* **2006**, *17*, 17–27.
[19] J. A. Hall, T. E. Thorgeirsson, J. Liu, Y. K. Shin, H. Nikaido, *J. Biol. Chem.* **1997**, *272*, 17610–17614.
[20] H. Taira, Y. Matsushita, K. Kojima, K. Shiraga, T. Hohsaka, *Biochem. Biophys. Res. Commun.* **2008**, *374*, 304–308.
[21] T. Watanabe, N. Muranaka, I. Iijima, T. Hohsaka, *Biochem. Biophys. Res. Commun.* **2007**, *361*, 794–799.
[22] S. Y. Chun, S. Strobel, P. Bassford, Jr., L. L. Randall, *J. Biol. Chem.* **1993**, *268*, 20855–20862.
[23] X. Ge, L. Tolosa, G. Rao, *Anal. Chem.* **2004**, *76*, 1403–1410.
[24] E. Nakata, Y. Koshi, E. Koga, Y. Katayama, I. Hamachi, *J. Am. Chem. Soc.* **2005**, *127*, 13253–13262.
[25] M. Fehr, W. B. Frommer, S. Lalonde, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 9846–9851.
[26] C. Kao, M. Zheng, S. Rudisser, *RNA* **1999**, *5*, 1268–1272.

Received: October 24, 2008
Published online on March 19, 2009

