J|A|C|S COMMUNICATIONS

Published on Web 10/30/2009

Photoactive Yellow Protein-Based Protein Labeling System with Turn-On Fluorescence Intensity

Yuichiro Hori, Hideki Ueno, Shin Mizukami, and Kazuya Kikuchi*

Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received June 12, 2009; E-mail: kkikuchi@mls.eng.osaka-u.ac.jp

There has been considerable interest in bioimaging technologies for the clarification of protein functions in living systems. So far, fluorescent proteins have made a significant contribution to research on protein expression, localization, and protein−protein interaction.[1] Although various fluorescent proteins (FP) are currently known,[2] it is difficult to visualize proteins in deep tissues, because proteins emitting near-infrared fluorescence, which can pass through thick tissues, are lacking. In addition, the FP size is large (~27 kDa), and therefore, there has been a strong demand for the generation of a smaller protein tag.[3] As an alternative technology, protein labeling systems such as Halo-tag and CLIP-tag have been developed.[4] In these methods, a specific pair of a protein tag and its ligand is employed for detecting proteins of interest. The advantages of these methods are that a variety of fluorescent dyes are potentially available as labeling reagents including near-infrared probes and that the tag proteins are labeled in controlled time. However, in these systems, free probes must be removed by washing cells or purifying cell lysate to distinguish the fluorescence of bound and unbound probes. To solve this issue, our group recently reported on a fluorogenic labeling method.[5] But these tag proteins are still large.[4,5] Although there are some other techniques for protein labeling,[6] they have drawbacks in bioorthogonality or require additional enzyme for protein modification. To overcome these limitations, we utilized photoactive yellow protein (PYP) as a tag protein and developed labeling reagents for this protein, including a fluorogenic probe, which would not require any procedure for removing the free fluorescent probe.

PYP is a small, water-soluble protein found in several purple bacteria.[7] It consists of 125 amino acids (14 kDa) and binds to a natural cofactor, CoA thioester of 4-hydroxycinnamic acid, through transthioesterification with Cys69.[8] In addition to the natural cofactor, it is known that PYP binds to the thioester derivative of 7-hydroxycoumarin-3-carboxylic acid,[9] which is a fluorescent compound. Importantly, PYP and its ligands do not exist in animal cells and, thus, it is expected that PYP expressed in those cells could be bioorthogonally labeled by its exogenous ligand without any cross reaction by endogenous factors. With regard to the design of a fluorogenic labeling system, 7-hydroxycoumarin has an interesting fluorescent property as follows. Previous work shows that a coumarin derivative linked with fluorescein through a flexible linker has no or little fluorescence properties because of the intramolecular association between the dyes and their dissociation triggers the increase in fluorescence intensity.[10] Based on this principle, we designed a probe that consists of 7-hydroxycoumarin-3-carboxylic acid thioester connected to fluorescein through an ethylene glycol linker (Figure 1). It is considered that the probe in the absence of PYP is not fluorescent due to the intramolecular interaction and, once the probe binds to PYP, the coumarin is dissociated from the fluorescein because the interaction between the coumarin and PYP prevents the close contact of two fluorophores.

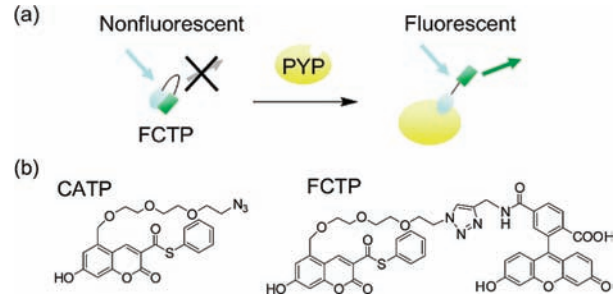

Figure 1. (a) Principle of fluorogenic labeling system based on PYP. (b) Structures of fluorescent probes, CATP and FCTP, for labeling PYP.

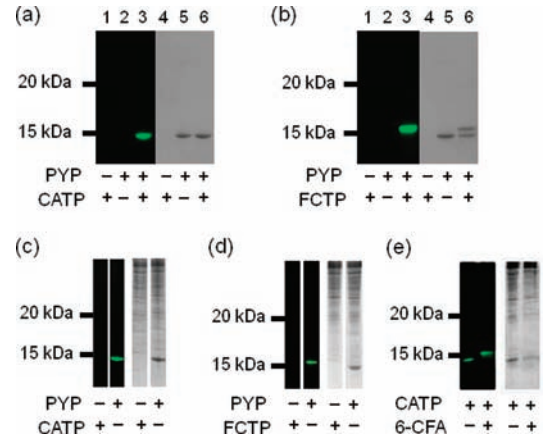

Figure 2. SDS-PAGE analyses of labeling reactions of PYP with probes, CATP and FCTP. Fluorescence and CBB-stained gel images are displayed on the left and right, respectively. PYP (10 μM) was reacted with CATP (25 μM) (a and c) or with FCTP (25 μM) (b and d). Images (c) and (d) represent the reactions in cell lysate. Image (e) represents the stepwise labeling of PYP (5 μM) with CATP (12.5 μM) and 6-CFA (200 μM) in cell lysate.

First, we synthesized CATP, which contains an azido group at the end of the linker (Figure 1b). Considering the steric hindrance, which is predicted from the structure of PYP with its natural cofactor,[11] the linker was introduced into the 5-position in the coumarin, because this position is assumed to be the 2-positon of the natural cofactor based on the structural similarity between these compounds. Then, fluorescein with an alkyne group (6-carboxy-fluorescein propalgylamide, 6-CFA) was conjugated to CATP by click chemistry to generate FCTP (Figure 1b). The probes were incubated with recombinant PYP purified from *E. coli*, and SDS-PAGE analysis was conducted to verify the binding of the probes to the protein (Figure 2a and b). In the absence of the probes, no fluorescence was detected in the gel. In the mixtures of the probes and PYP, fluorescent bands appeared, indicating that the probes bind to PYP. Interestingly, the reaction of FCTP and PYP yielded

10.1021/ja904800k CCC: $40.75

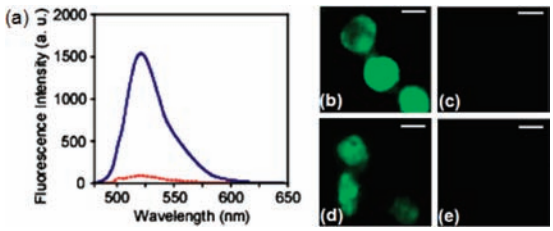

Figure 3. (a) Fluorescence spectra of FCTP (8 μM) in the absence (red dashed line) or presence (blue line) of PYP (5 μM). (b−e) Fluorescent live-cell imaging. Images (b, d) or (c, e) represent the cells that expressed or did not express PYP-PDGFRtm, respectively. The cells after incubation with CATP (5 μM; b, c) or FCTP (20 μM; d, e) are shown. Scale bars = 10 μm.

a slowly migrating band, which is regarded as FCTP-bound PYP because of its fluorescence. The binding of PYP and the probes was also confirmed by MALDI-TOF MS (Figure S1). The addition of CATP or FCTP to PYP gave the mass value of PYP bound to the individual probe, in which thiophenyl ester is replaced by thioester of Cys in the protein. The results indicate that the probes covalently bind to PYP through transthioesterification.

The binding specificity of the probes toward PYP was investigated. Labeling reactions of purified PYP were carried out in the lysate prepared from HEK293T cells. Figure 2c and d show that a single fluorescent band was detected only in the reaction mixture of PYP and each probe, confirming that PYP is specifically labeled by CATP or FCTP under this experimental condition. The influence of free thiols on the labeling reaction was also examined. The presence of a physiological concentration of glutathione (up to 10 mM) did not affect the labeling reactions (Figure S2).[13] Furthermore, the azido moiety of CATP would allow additional labeling of PYP with the second probe by click chemistry and could expand the range of the applications of this system. After the reaction of PYP and CATP in the cell lysate, 6-CFA was added to the reaction mixture in the presence of Cu^{2+} and tris(2-carboxyethyl)phosphine. Electrophoresis revealed a slowly migrating band, as is the case with the FCTP-bound PYP (Figure 2e). There were no newly appearing bands, demonstrating that the stepwise labeling reaction is quite specific.

To examine the fluorogenic properties of FCTP, the fluorescent spectra of the probes were measured (Figure 3a). In the absence of PYP, the fluorescence intensity of FCTP is very weak, suggesting that the coumarin and fluorescein dyes in the probe associate with each other. On the other hand, the binding of PYP and the probe leads to a dramatic increase in the fluorescence intensity. This increase is approximately 20-fold after 24 h of incubation. The result indicates that the coumarin dissociates from the fluorescein due to the formation of the complex between PYP and the probe. We then characterized the binding kinetics of PYP with CATP and FCTP by size exclusion chromatography and fluorescence measurement, respectively (Figures S3 and S4; see Supporting Information for the detailed procedure). The CATP reaction was almost complete within 2 h, consistent with a previous report, demonstrating the binding kinetics of a natural ligand to PYP.[12] In contrast, the binding of FCTP to PYP was slow, requiring more than 24 h to complete the reaction. One probable reason for this difference is that the intramolecular interaction in FCTP could influence its binding kinetics.

Finally, cell labeling experiments were conducted. HEK293T cells expressing PYP-PDGFRtm (the fusion protein of PYP and a transmembrane domain of platelet-derived growth factor receptor) on the cell membrane were prepared. CATP or FCTP was incubated with the cells in culture media. Fluorescence microscopy showed that fluorescent labeling by both probes occurred in the cells expressing PYP-PDGFRtm (Figures 3b and S5). No fluorescence was observed in the cells that did not express PYP-PDGFRtm, demonstrating that PYP is specifically labeled on the cell membrane by both probes. During the experiments, we noticed that CATP was cell-permeable. Therefore, intracellular imaging with CATP was also performed. After the labeling reaction of CATP and the cells expressing maltose binding protein-fused PYP (MBP-PYP) in cytosol, fluorescence was observed only in the cells expressing MBP-PYP, and not in the nonexpressing cells (Figure S5). This result clearly shows that CATP allows specific labeling of PYP inside living cells.

In conclusion, we have developed a protein labeling system, based on a small tag protein, PYP, and its fluorescent probes. The live-cell imaging and specific labeling of PYP were achieved by using CATP and FCTP. CATP has dual functions as a fluorescent probe and a chemical handle for two-step labeling. More importantly, FCTP shows fluorogenic characteristics, allowing the identification of the probe bound to its tag protein. These properties offer a more sophisticated application of this system to protein imaging studies.

Acknowledgment. This work was supported by MEXT of Japan. We thank Prof. Klaas J. Hellingwerf for providing the plasmid encoding PYP and Dr. Aya Fukuda for giving the plasmid for mammalian expression.

Supporting Information Available: Experimental procedures and supplemental results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Zaccolo, M. *Circ. Res.* **2004**, *94*, 866–873. (b) VanEngelenburg, S. B.; Palmer, A. E. *Curr. Opin. Chem. Biol.* **2008**, *12*, 60–65.
(2) (a) Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. *Nat. Methods* **2005**, *2*, 905–909. (b) Pakhomov, A. V.; Martynov, V. I. *Chem. Biol.* **2008**, *15*, 755–764.
(3) (a) Zhou, Z.; Koglin, A.; Wang, Y.; McMahon, A. P.; Walsh, C. T. *J. Am. Chem. Soc.* **2008**, *130*, 9925–9930. (b) Chen, I.; Ting, A. Y. *Curr. Opin. Biotechnol.* **2005**, *16*, 35–40.
(4) (a) Los, G. V.; Wood, K. *Methods Mol. Biol.* **2007**, *356*, 195–208. (b) Gautier, A.; Juillerat, A.; Heinis, C.; Corrêa, I. R., Jr.; Kindermann, M.; Beaufils, F.; Johnsson, K. *Chem. Biol.* **2008**, *15*, 128–136.
(5) Mizukami, S.; Watanabe, S.; Hori, Y.; Kikuchi, K. *J. Am. Chem. Soc.* **2009**, *131*, 5016–5017.
(6) (a) O'Hare, H. M.; Johnsson, K.; Gautier, A. *Curr. Opin. Struct. Biol.* **2007**, *17*, 488–494. (b) Wu, P.; Shui, W.; Carlson, B. L.; Hu, N.; Rabuka, D.; Lee, J.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3000–3005. (c) Fernández-Suárez, M.; Baruah, H.; Martínez-Hernández, L.; Xie, K. T.; Baskin, J. M.; Bertozzi, C. R.; Ting, A. Y. *Nat. Biotechnol.* **2007**, *25*, 1483–1487. (d) Nonaka, H.; Tsukiji, S.; Ojida, A.; Hamachi, I. *J. Am. Chem. Soc.* **2007**, *129*, 15777–15779. (e) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063–6076.
(7) Kamiuchi, M.; Hara, M. T.; Stalcup, P.; Xie, A.; Hoff, W. D. *Photochem. Photobiol.* **2008**, *84*, 956–969.
(8) Kyndt, J. A.; Meyer, T. E.; Cusanovich, M. A.; Van Beeumen, J. J. *FEBS Lett.* **2002**, *512*, 240–244.
(9) van der Horst, M. A.; Arents, J. C.; Kort, R.; Hellingwerf, K. J. *Photochem. Photobiol. Sci.* **2007**, *6*, 571–579.
(10) Takakusa, H.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *Anal. Chem.* **2001**, *73*, 939–942.
(11) Anderson, S.; Crosson, S.; Moffat, K. *Acta Crystallogr., Sect. D* **2004**, *60*, 1008–1016.
(12) Imamoto, Y.; Ito, T.; Kataoka, M.; Tokunaga, F. *FEBS Lett.* **1995**, *374*, 157–160.
(13) Wu, G.; Fang, Y. Z.; Yang, S.; Lupton, J. R.; Turner, N. D. *J. Nutr.* **2004**, *34*, 489–492.

JA904800K