J|A|C|S COMMUNICATIONS

Published on Web 03/18/2009

Covalent Protein Labeling Based on Noncatalytic β-Lactamase and a Designed FRET Substrate

Shin Mizukami, Shuji Watanabe, Yuichiro Hori, and Kazuya Kikuchi*

Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received October 20, 2008; E-mail: kkikuchi@mls.eng.osaka-u.ac.jp

Fluorescence microscopy is one of the most common techniques employed in the field of life science. With the rapid progress that has been achieved with regard to optical systems, fluorescent proteins (FPs) have acquired important roles for fluorescence microscopy experiments. In order to visualize the localization and behavior of particular proteins of interest, FPs such as green fluorescent protein (GFP) have conventionally been used.[1] More recently, techniques for labeling proteins with small molecules have attracted the attention of many life scientists because they can extend the range of natural FPs, for example, by incorporating near-infrared fluorescent dyes, MRI contrast agents, or biofunctional molecules such as biotin. Several approaches for modifying proteins with small molecules have been commercialized, including methods based on the tetracysteine tag,[2] HaloTag,[3] and SNAP-tag.[4] Other protein labeling methods involving the use of biotin ligase,[5] transglutaminase,[6] hexahistidine,[7] tetra-aspartic acid,[8] etc. have also been reported. Among the abovementioned labeling methods, only the tetracysteine tag exhibits fluorogenic properties. In the other labeling methods, it is necessary to wash the treated cells prior to microscopic measurements to eliminate background fluorescence. Thus, new labeling techniques that satisfy the dual criteria of specificity and fluorogenicity are desirable.

In this paper, we report a specific protein labeling system with an off-on fluorescence switch. It involves covalent modification of a genetically engineered hydrolytic enzyme with a rationally designed fluorogenic probe that exploits the principle of fluorescence resonance energy transfer (FRET). Using this system, we can achieve specific and fluorogenic protein labeling under physiological conditions.

First, we designed the tag protein. Plant or bacterial proteins are preferably used to achieve bioorthogonal labeling in mammalian cells. We focused on β-lactamase as the candidate tag because β-lactamases are small bacterial enzymes that hydrolyze antibiotics containing a β-lactam structure and have no endogenous counterpart among eukaryotic cells.[9] β-Lactamase has been widely used as a reporter enzyme for examining gene expression in living mammalian cells.[10] Class A β-lactamases such as the 29 kDa TEM-1[11] have been extensively investigated with regard to their structures, enzyme reaction kinetics, substrate specificity, inhibitors, etc.[12] The reaction of TEM-1 with β-lactams involves acylation and deacylation steps (Scheme 1). In the acylation step, Ser70 attacks the amide bond of the β-lactam ring to form an intermediate acyl-enzyme complex (ES*). In the deacylation step, an activated water molecule hydrolyzes the ester bond of the intermediate to yield the product. Previous studies have shown that Glu166 is essential for the deacylation step[13] and that the E166N mutant of TEM-1 (^{E166N}TEM) accumulates the acyl-enzyme intermediate by markedly slowing deacylation (k_3) relative to acylation (k_2).[14] We hoped to exploit the properties of the ^{E166N}TEM mutant to covalently attach a fluorescent substrate to β-lactamase.

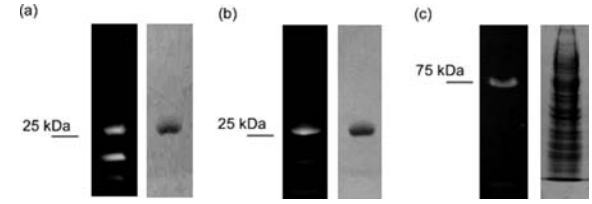
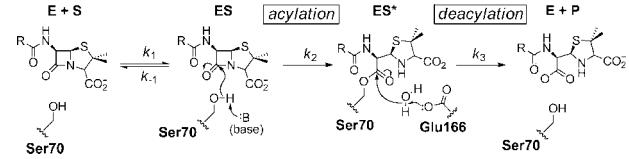
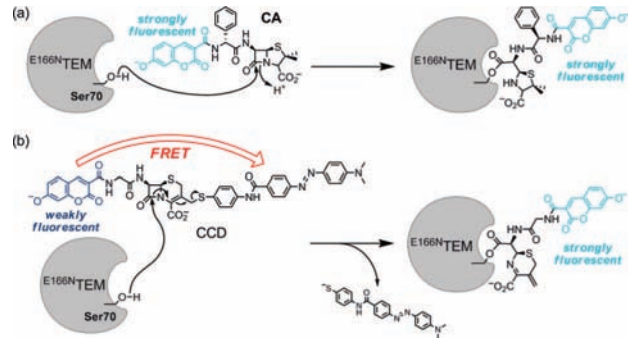


Figure 1. (a, b) Fluorescence (left) and CBB-stained (right) gel images of ^{E166N}TEM after incubation with (a) CA and (b) CCD. (c) Fluorescence and CBB-stained gel image of MBP-^{E166N}TEM mixed with HEK293T cell lysate after incubation with CCD.

Scheme 1. Mechanism of β-Lactam Cleavage by Class A β-Lactamases; (E) Enzyme, (S) Substrate, and (P) Product



Scheme 2. Structures and Labeling Mechanisms of the Fluorescent Probes (a) CA and (b) CCD



To investigate the feasibility of fluorescently labeling ^{E166N}TEM under physiological conditions, we designed and synthesized a penicillin-based fluorescent probe, coumarinyl ampicillin (CA). The labeling scheme is illustrated in Scheme 2a. Since CA contains 7-hydroxycoumarin, successfully labeled ^{E166N}TEM should exhibit cyan fluorescence. ^{E166N}TEM was incubated with CA in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C, and protein labeling was assessed by SDS-PAGE. Fluorescent proteins were detected by irradiating the gels with UV light at 365 nm. When purified ^{E166N}TEM was mixed with CA, a protein band of ∼29 kDa was observed that exhibited cyan fluorescence (Figure 1a); Coomassie Brilliant Blue (CBB) staining confirmed that this band corresponded to ^{E166N}TEM. In contrast, when wild-type (WT) TEM-1 was incubated with CA, no cyan fluorescence was seen (Figure S1a). Although CA successfully labels ^{E166N}TEM, other fluorescent bands were also observed on the gel. Since these bands were also seen when only

10.1021/ja8082285 CCC: $40.75

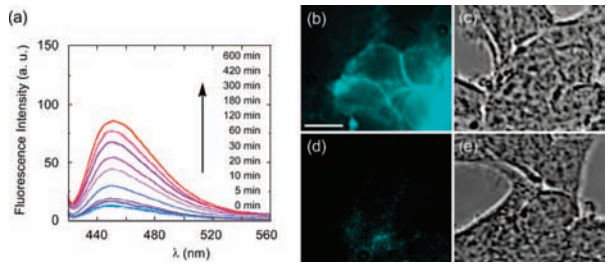


Figure 2. (a) Time-dependent emission spectra ($\lambda_{ex} = 410$ nm) of CCD (1 μM) in the presence of ^{E166N}TEM in 100 mM HEPES buffer (pH 7.4) containing 0.1% DMSO at 25 °C. (b−e) Optical microscopic images of CCD-labeled HEK293T cells expressing ^{E166N}TEM-EGFR (b,c) and EGFR (d,e), labeled with 5 μM CCD. (b,d) Fluorescence microscopic images, excitation at 410 nm. (c,e) phase contrast microscopic images. Scale bar: 10 μm.

CA was electrophoresed (Figure S2), a washing procedure should be performed before observation under a fluorescence microscope.

Next, we designed and synthesized CCD (Scheme 2b), a fluorescence off−on labeling probe. This molecule has three main components: 7-hydroxycoumarin, cephalosporin, and 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL). Since the absorption spectrum of DABCYL substantially overlaps with the emission spectrum of 7-hydroxycoumarin, the fluorescence of CCD would be expected to be largely quenched by intramolecular FRET from coumarin to DABCYL. Based on related probes of β-lactamase activity,[10] cleavage of the β-lactam of CCD by ^{E166N}TEM should result in covalent attachment of the coumarin to the protein with concomitant release of the DABCYL moiety as shown in Scheme 2b. After loss of the DABCYL group, the cyan fluorescence of coumarin should be restored by cancelation of FRET.

The fluorescence spectrum of CCD confirmed that the coumarin fluorescence was almost completely quenched because of FRET. The fluorescence quantum yield of CCD was 0.006, which is much lower than that of CA ($\Phi = 0.40$). When CCD was incubated with ^{E166N}TEM, the fluorescence increased considerably in a time-dependent manner (Figure 2a). This indicates that ^{E166N}TEM cleaved the β-lactam of CCD and eliminated the DABCYL group. When the DABCYL group was completely eliminated by WT TEM-1, the fluorescence signal increased approximately 30-fold. The apparent rate of reaction between CCD and ^{E166N}TEM was approximately 80-fold slower than that of the reaction between CCD and WT TEM-1 (Figure S3), probably because the mutation at E166 decreases the acylation rate (k_2) somewhat.[15]

CCD specifically labels ^{E166N}TEM, as demonstrated by incubation of the probe molecule with both ^{E166N}TEM and WT TEM-1 in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C, followed by SDS-PAGE analysis. As shown in Figure 1b, only the band corresponding to ^{E166N}TEM exhibited cyan fluorescence; no fluorescence was associated with WT TEM-1 (Figure S1b). In contrast to CA, unreacted CCD yielded considerably weaker fluorescence on the gel. In MALDI-TOF MS analyses of the samples, the molecular mass peak for the protein−probe adduct was only detected when ^{E166N}TEM was incubated with CCD (Figure S4).

This system can therefore be used to label target proteins in a biological medium. For example, we fused ^{E166N}TEM to maltose binding protein (MBP, 42 kDa), mixed the purified MBP—^{E166N}TEM construct with HEK293T cell lysate, and incubated the mixture with CCD at 25 °C for 45 min. SDS-PAGE analysis revealed that fusion protein was efficiently and selectively labeled with the fluorogenic probe (Figure 1c).

Finally, we investigated specific labeling of target proteins displayed on the surface of living cells. ^{E166N}TEM was fused to the N-terminus of epidermal growth factor receptor (EGFR), a membrane associated protein, and the construct was produced in HEK293T cells. After treatment with CCD (see Supporting Information), the cells were examined under a fluorescence microscope. Only cells producing the ^{E166N}TEM-EGFR fusion protein emitted cyan fluorescence as a consequence of specific labeling by the probe (Figure 2b−e).

In conclusion, we have developed a novel protein labeling system that combines a genetically modified β-lactamase with low molecular weight fluorogenic β-lactam probes. Through appropriate probe design, we succeeded in labeling proteins with a sensitive fluorophore in vitro and on living cells. In principle, this system does not require washing procedures to remove the unreacted probes after labeling. Furthermore, since the ^{E166N}TEM tag protein is absent in mammalian cells, it can be used for the specific labeling of proteins in higher eukaryotes. We anticipate that this labeling system will find wide application in the field of life science.

Acknowledgment. We thank Dr. Shahriar Mobashery at the University of Notre Dame for kindly providing TEM-1 plasmid. We also thank Dr. Robert E. Campbell at Alberta University, Dr. Gregor Zlokarnik at Vertex Pharmaceuticals, and Dr. Donald Hivert at ETH Zürich for helpful discussions. S.W. acknowledges a Global COE Fellowship of Osaka University. This work was supported in part by MEXT of Japan.

Supporting Information Available: Detailed experimental procedures and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chudakov, D. M.; Lukyanov, S.; Lukyanov, K. A. *Trends Biotechnol.* **2005**, *23*, 605–613.
(2) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269–272.
(3) Los, G. V.; et al. *ACS Chem. Biol.* **2008**, *3*, 373–382.
(4) Keppler, A.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86–89.
(5) Chen, I.; Howarth, M.; Lin, W.; Ting, A. Y. *Nat. Methods* **2005**, *2*, 99–104.
(6) Lin, C.-W.; Ting, A. Y. *J. Am. Chem. Soc.* **2006**, *128*, 4542–4543.
(7) Hauser, C. T.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3693–3697.
(8) Ojida, A.; Honda, K.; Shinmi, D.; Kiyonaka, S.; Mori, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2006**, *128*, 10452–10459.
(9) Waley, S. G. *In the chemistry of β-lactams*; Page, M. I., Ed.; Chapman & Hall: London, 1992; p 198.
(10) (a) Moore, J. T.; Davis, S. T.; Dev, I. K. *Anal. Biochem.* **1997**, *247*, 203–209. (b) Zlokarnik, G.; Negulescu, P. A.; Knapp, T. E.; Mere, L.; Burres, N.; Feng, L.; Whitney, M.; Roemer, K.; Tsien, R. Y. *Science* **1998**, *279*, 84–88. (c) Gao, W.; Xing, B.; Tsien, R. Y.; Rao, J. *J. Am. Chem. Soc.* **2003**, *125*, 11146–11147. (d) Campbell, R. E. *Trends Biotechnol.* **2004**, *22*, 208–211. (e) Xing, B.; Khanamiryan, A.; Rao, J. *J. Am. Chem. Soc.* **2005**, *127*, 4158–4159.
(11) Sutcliffe, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3737–3741.
(12) Matagne, A.; Lamotte-Blasseur, J.; Frére, J.-M. *Biochem. J.* **1998**, *330*, 581–598.
(13) Guillaume, G.; Vanhove, M.; Lamotte-Brasseur, J.; Ledent, P.; Jamin, M.; Joris, B.; Frére, J.-M. *J. Biol. Chem.* **1997**, *272*, 5438–5444.
(14) Adachi, H.; Ohta, T.; Matsuzawa, H. *J. Biol. Chem.* **1991**, *266*, 3186–3191.
(15) Vijayakumar, S.; Ravishanker, G.; Pratt, R. F.; Beveridge, D. L. *J. Am. Chem. Soc.* **1995**, *117*, 1722–1730.

JA8082285