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Design and Synthesis of an Enzyme Activity-Based Labeling Molecule with Fluorescence Spectral Change

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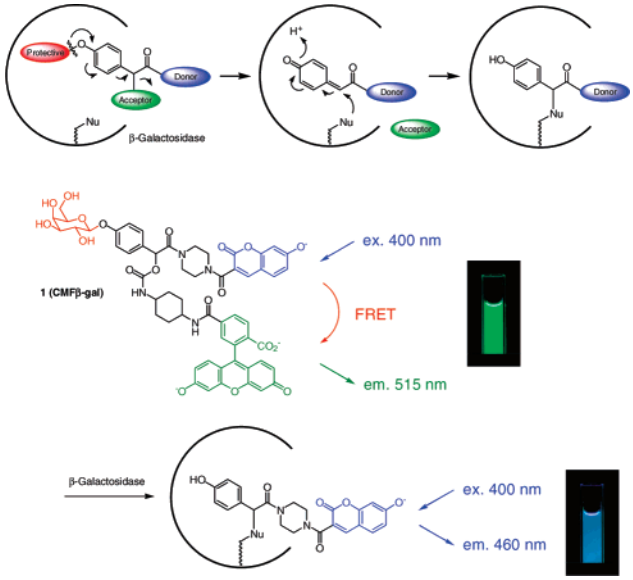
Since proteins play central roles in a variety of cellular events, the study of protein dynamics is critical to an understanding of sophisticated processes of life. In recent years, chemistry-based strategies to modify, control, and monitor specific proteins have proved useful.[1−4] As represented by FlAsH that binds to a tetracysteine motif[5] and benzylguanine derivatives that bind to O^6-alkylguanine−DNA alkyltransferase,[6] techniques of covalent labeling of a specific tag protein with small-molecular dyes are effective for monitoring dynamic behaviors of proteins in living cells. However, one of the problems in monitoring protein dynamics with these techniques is the delay before detection of the target protein, owing to the need to wash out excess unreacted probe. It is desirable to develop a protein labeling technique that visualizes the process of the labeling reaction so that the labeled protein can be detected immediately at the point of labeling, that is, in the presence of unreacted probe.

In this paper, we describe a novel protein labeling probe that overcomes this problem by exhibiting a fluorescence wavelength change. With a probe whose fluorescence wavelength changes during the course of the labeling reaction, fluorescence of the labeled protein can be observed separately from that of the unreacted probe, so immediate detection and precise quantification of the target protein should be possible by monitoring the emission wavelength shift.

The design of the labeling reaction was based on quinone methide chemistry. Controlled formation and selective reaction of quinone methide have proved to be useful in chemically modifying cellular components such as nucleotides and proteins.[7−10] An advantage of this reaction is that formation of quinone methide entails bond scission, thereby changing the basic structure of the probe. In fluorescent probes based on fluorescence resonance energy transfer (FRET), structural change of the probe can be converted to a change of efficiency of FRET between two fluorophores.[11,12] The designed probe has two fluorophores that are expected to exhibit FRET, that is, 7-hydroxycoumarin as an energy donor and fluorescein as an energy acceptor. In the intact probe, these two fluorophores are positioned close to each other and FRET occurs efficiently. Upon labeling, the FRET efficiency decreases owing to loss of the donor fluorescein moiety as a leaving group. The change of FRET efficiency is reflected in an increase of fluorescence of the donor 7-hydroxycoumarin and a decrease of that of the acceptor fluorescein. By means of ratiometric measurement, the reaction rate can be monitored accurately.

Scheme 1. The Design of CMFβ-gal



In quinone methide-forming molecules reported to date, halogens are mostly used as a leaving group,[7−10] but a leaving group with an appropriate structure is required for our purpose. This led us to design a probe with carbamate as a leaving group.

A good trigger for quinone methide formation is an enzymatic reaction that cleaves a phenol protective group to give a free phenol. We chose β-galactosidase from *Escherichia coli*, encoded by the *LacZ* gene,[13] as a target for labeling since its high turnover number for β-galactopyranosyl-protected phenols (1.8×10^5 s^{-1} for 2-nitrophenyl β-galactopyranoside[14]), its high substrate specificity for the glycon unit, and its wide tolerance for the aglycon unit are desirable features for a target of our probes.

The probe, named CMFβ-gal (coumarin−mandelate−fluorescein β-galactopyranosyl), was synthesized in 10 steps from commercially available 4-hydroxymandelic acid. As shown in the Supporting Information, a control compound CMfluorideβ-gal (coumarin−mandelate−fluoride β-galactopyranoside), with fluoride as a leaving group, was also synthesized for comparison of reactivity.

CMFβ-gal is stable in aqueous buffer solution (pH 7.4, 37 °C) for >24 h. The emission spectrum of CMFβ-gal in an aqueous buffer solution excited at 400 nm (peak excitation wavelength of 7-hydroxycoumarin) is shown in Figure 1.

Initially, emission of 7-hydroxycoumarin at around 460 nm was hardly observed, while a strong emission of fluorescein was seen at around 515 nm. This demonstrates that energy transfer proceeds efficiently in the intact probe, and FRET efficiency was calculated

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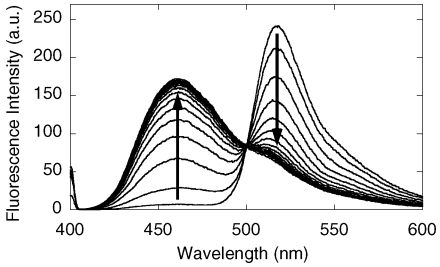

Figure 1. The emission spectra of a 1.0 μM solution of CMFβ-gal after the addition of β-galactosidase (11.6 μg·mL^{-1}) in phosphate buffer (10 mM, pH 7.4) at 37 °C. The spectra were measured at every 4 min after the addition of β-galactosidase, λ_{ex} = 400 nm.

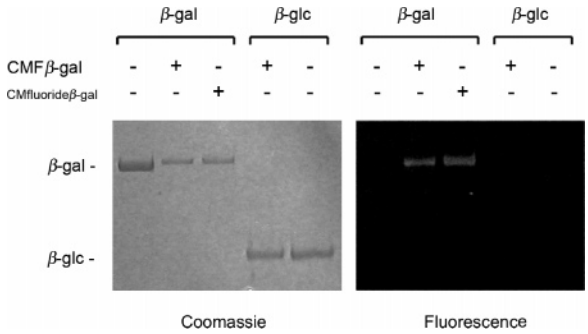

Figure 2. The result of SDS−PAGE (see Supporting Information for details of sample preparation). Left: image of Coomassie brilliant blue-stained gel. Right: fluorescence image of the gel under illumination at 365 nm from a UV lamp.

to be >93%. When β-galactosidase was added to the solution, a rapid increase in the emission of 7-hydroxycoumarin and a corresponding rapid decrease in the emission of fluorescein were observed. Monitoring of the increase of the ratio of fluorescence intensities at 460 and 515 nm allowed accurate estimation of the β-galactosidase concentration (see Supporting Information). After fluorescence change of reaction mixture had ceased, β-galactosidase was purified and its spectrum was measured to confirm that CMFβ-gal had indeed acted as a labeling probe of β-galactosidase. As shown in the Supporting Information, purified β-galactosidase exhibited the characteristic absorbance and fluorescence spectra of 7-hydroxycoumarin. These results were the same as that obtained with a control compound, CMfluorideβ-gal. In contrast, no labeling occurred when the probe was mixed with a control protein, β-glucosidase, instead of β-galactosidase. The labeled protein could also be detected with SDS−PAGE. As shown in Figure 2, fluorescence of 7-hydroxycoumarin was observed at the spot of β-galactosidase labeled with the probe, while it was not observed at the spot of β-glucosidase.

Finally, CMFβ-gal was used for ratiometric imaging in *LacZ*-positive or *LacZ*-negative cells to confirm that it can detect β-galactosidase expression in living cells (Figure 3). After microinjection of the probe, slight leakage occurred, but the increase in the emission of 7-hydroxycoumarin was clearly observed in *LacZ*-positive cells, while it was not apparent in *LacZ*-negative cells (see Supporting Information).

In conclusion, we have established a novel design strategy for quinone methide chemistry-based protein labeling probes, with carbamate as a leaving group. On the basis of this design, we

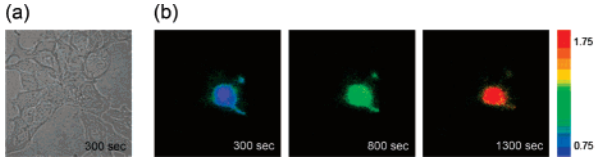

Figure 3. Bright-field transmission (a) and ratiometric images (b) of CMFβ-gal-injected *LacZ*-positive HEK293 cells at 300, 800, and 1300 s after microinjection. See Supporting Information for details.

synthesized a β-galactosidase labeling probe, CMFβ-gal, the labeling reaction of which can be monitored in terms of the change of fluorescence wavelength. This is the first report of a protein labeling probe which features a change of fluorescence wavelength upon reaction, allowing the labeled protein to be detected even in the presence of unreacted probe. The general probe design allows a wide tolerance in the selection of fluorophore pairs as well as protective groups for various enzymes. In applying this system to the other target enzymes, one possible limitation is that the feasibility of the labeling may depend on the structure of the protein, especially on the location of nucleophilic amino acids to be labeled.[9,10] Though, in future work, we think it would be possible to develop a range of probe and target enzyme pairs that serve as powerful tools in the study of protein dynamics in living cells.

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Supporting Information Available: Synthesis, experimental details, and characterization of CMFβ-gal and CMfluorideβ-gal, and additional experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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