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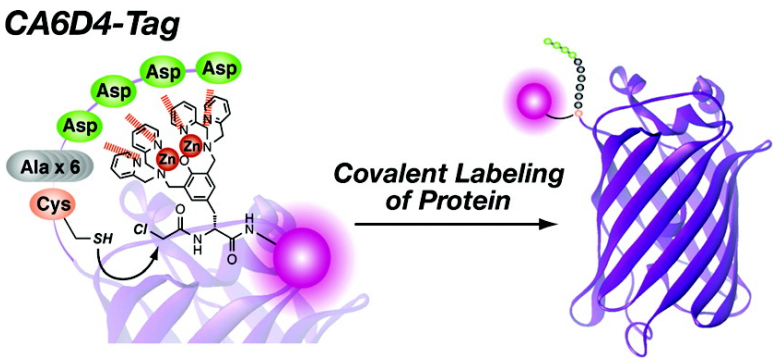

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Non-enzymatic Covalent Protein Labeling Using a Reactive Tag

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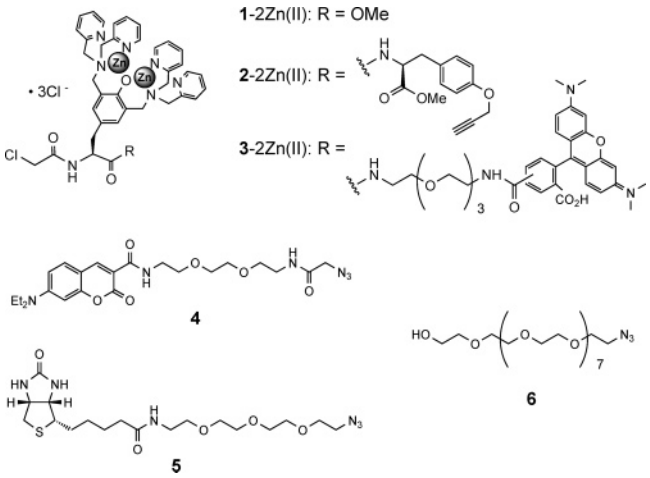
Labeling proteins with functional molecules such as luminescent markers or affinity tags is an approach widely used in protein research.[1] While a variety of protein labeling methods using specific ligand−protein interactions are currently available,[2] the advantage of irreversible covalent labeling is that its permanence allows unambiguous analyses of the protein after labeling. Recently, enzyme-catalyzed protein labeling methods have been developed, in which a substrate analogue can be covalently attached to an enzyme, protein domain, or peptide tag fused to a target protein.[3] In contrast, non-enzymatic covalent labeling methods which have a sufficient chemo- and site-selectivity have been less successful,[4] despite not needing a large enzyme/protein domain and having a wide range of labeling reagents and reaction conditions.

We describe herein a method for the non-enzymatic selective covalent labeling of tag-fused proteins with small probes, on the basis of their molecular recognition and chemical reaction. We have recently developed a complementary recognition pair of the short tetraaspartate sequence (D4-tag; DDDD) and the Zn(II)-DpaTyr probe for the noncovalent labeling of proteins.[5] Introduction of a mildly reactive electrophile on the probe and a nucleophilic thiol on the tag achieved the rapid and specific covalent labeling of a tag-fused protein. (Figure 1). The advantages of this method over the previous noncovalent method are (i) various artificial molecules can be permanently incorporated into the tag-fused protein, (ii) postlabeling analyses such as SDS-PAGE or blotting can be performed conveniently, and (iii) the labeling can be performed in crude conditions such as protein mixtures, cell lysate, and inside of cells.

For the covalent bond formation between the D4-tag peptide and Zn(II)-DpaTyr, a nucleophilic reaction of α-haloketone with a cysteine thiol group was employed.[6] We prepared *N*-α-chloroacetyl Zn(II)-DpaTyr (**1**-2Zn(II)) as a thiol-reactive probe and a series of the D4-tag peptides Cys-Ala$_n$-Asp$_4$ (CAnD4, n = 0, 2, 4, 6, 8) possessing one Cys group at the various distal positions from the D4 sequence, and evaluated their reactivities. Upon addition of **1**-2Zn(II) (20 μM) to an aqueous solution of CA6D4 peptide (10 μM in 50 mM HEPES buffer, pH 7.2, 100 mM NaCl), rapid reaction occurred at 20 °C to give the covalent adduct. This was confirmed by MALDI-TOF mass analysis, which showed the appearance of a new peak of the covalent product (MW: 2161.83) and the concomitant disappearance of the original CA6D4 peptide peak (MW: 1376.56) (Supporting Information, Figure S1). HPLC analysis showed that the CA6D4 peptide was almost completely consumed within 1 h (Figure 2a, Figure S2). The reaction did not proceed in the case of the SA6D4 peptide in which Cys was replaced with Ser (Figure 2a), confirming that the covalent bond formation occurred at the Cys thiol group of the CA6D4 peptide. The covalent product was not observed in the presence of 3 mM



of pyrophosphate (PPi), a strong binder for Zn(II)-DpaTyr even after 2 h (Figure 2b). These results indicate that nucleophilic attack of the Cys residue on the chloroacetyl group is facilitated by the tight binding between **1**-2Zn(II) and the peptide owing to the metal-D4 coordination interaction (K_d = 1.4 μM in 50 mM HEPES buffer, pH 7.2).

We applied this system to labeling the EGFP (enhanced green fluorescent protein) fused to the CA6D4 tag at its *C*-terminal (CA6D4-EGFP). When the probe **2**-2Zn(II) (20 μM) was added to an aqueous solution of CA6D4-EGFP (5 μM in 50 mM HEPES 100 mM NaCl, 1 mM DTT, pH 7.2), the labeling took place rapidly as shown in Figure 3a. The MALDI-TOF mass data indicated that the new peak corresponding to the covalent adduct of CA6D4-EGFP and **2**-2Zn(II) appeared with a concomitant disappearance of the peak of CA6D4-EGFP.[7] The labeling reaction was complete within 15 min (Figure 3a and S3), whereas no change in the mass spectrum was observed in the case of EGFP lacking a CA6D4 tag (data not shown). These results indicate that the labeling site of the CA6D4-EGFP is the Cys residue of the attached reactive tag. The high reactivity and selectivity of **2**-2Zn(II) toward CA6D4-EGFP was demonstrated by the labeling reaction proceeding smoothly, even in the presence of 1 mM of dithiothreitol (DTT), a potentially competitive reactant to **2**-2Zn(II). The labeling reaction was followed by the modification of the propargyl unit of **2**-2Zn-(II) with coumarin azide **4** by the Huisgen reaction[8] to give the fluorophore-appended EGFP, detected by in-gel fluorescence analysis (Figure 3b).[9] Other azide derivatives such as biotin **5** or poly(ethylene glycol) **6** also underwent the Huisgen reaction to yield the corresponding modified EGFP (Figure S4), indicating the general applicability of the present reactive tag system to the attachment of artificial functional groups to a target protein.

To evaluate the specificity of the reaction, the labeling experiment was conducted using a mixture of five soluble proteins such as bovine serum albumin (BSA), glutathione *S*-transferase (GST),

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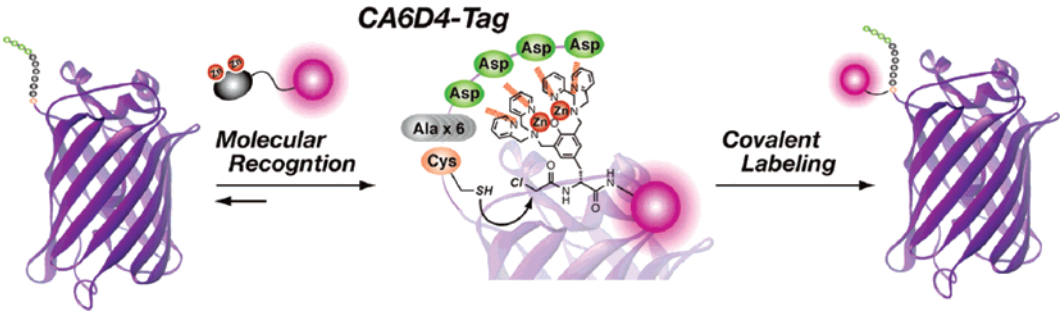

Figure 1. New covalent protein labeling method reported herein.

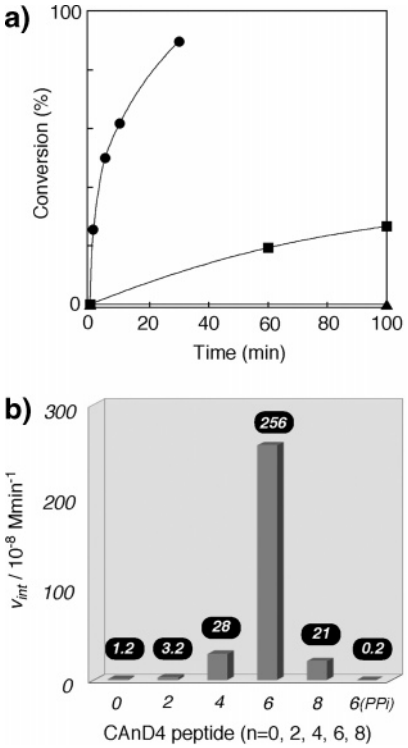

Figure 2. (a) Time trace of the labeling reaction of **1**-2Zn(II) with CA6D4 (●), CA2D4 (■), and SA6D4 peptide (▲). (b) Summary of the initial rate (v_{int}, M min^{-1}) of the labeling reaction of **1**-2Zn(II) with the CAnD4 peptide (n = 0, 2, 4, 6, 8); 6(PPi) means the reaction with the CA6D4 peptide in the presence of 3 mM of pyrophosphate (PPi). Reaction conditions: 20 μM **1**-2Zn(II), 10 μM tag peptide in 50 mM HEPES, 100 mM NaCl, pH 7.2, 20 °C.

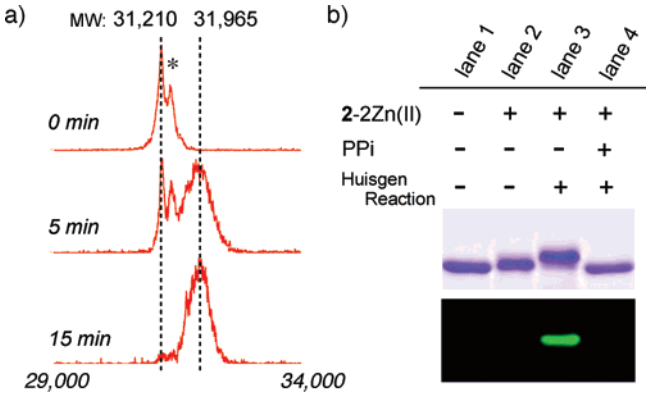

Figure 3. Covalent labeling of the CA6D4-tagged EGFP with **2**-2Zn(II). (a) MALDI-TOF mass analysis of the labeling reaction. Reaction conditions: 20 μM **2**-2Zn(II), 5 μM CA6D4-EGFP in 50 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.2, 20 °C. (The asterisk (∗) is the peak of CA6D4-EGFP + matrix) (b) SDS-PAGE analysis of the labeling reaction using Coomassie staining (upper) and in-gel fluorescence visualization (lower). The analysis was performed after Huisgen reaction with coumarin azide **4**. Experimental details of the Huisgen reaction are described in Supporting Information.

hemoglobin (HG), thioredoxin (TR), and CA6D4-EGFP, all with free cysteine thiol groups on their surfaces (Figure 4a). Incubation of **2**-2Zn(II) (20 μM) with the protein mixture and the subsequent Huisgen reaction afforded a single fluorescence band corresponding to the coumarin-labeled-EGFP (Figure 4a, lane 3), showing that the Cys in the CA6D4 tag reacts uniquely with **2**-2Zn(II) in preference to free thiol groups of the other proteins. The specificity was also evaluated using another set of the protein mixture, which included phosphorylated ovalbumin (OVA), ribonuclease (RNase), β-galactosidase (β-Gal), BSA, and CA6D4-EGFP (Figure 4b).

The in-gel fluorescence analysis again showed that only a single protein, CA6D4-EGFP was labeled with **2**-2Zn(II) (Figure 4b, lane 3). The inhibition of the labeling by PPi was observed in both mixture samples. These results demonstrate that the complementary tag−probe binding efficiently induced the selective labeling of the tag-fused EGFP among the various proteins.

The selective labeling of a tag-fused protein was examined using cruder samples such as cell lysate and live cell (Figure 5a). Incubation of *E. coli* cell lysate overexpressing the tag-fused maltose binding protein (CA6D4-MBP, ca. 5 μM) with 20 μM of the rhodamine-tethered probe **3**-2Zn(II) afforded the labeled MBP, which was detected as a single fluorescence band in the in-gel fluorescence analysis (Figure 5a, lane 3). In contrast, the fluorescent band was not detected for another recombinant MBP that possessed a free Cys at its *C*-terminal but lacked the D4 sequence nearby (C-MBP, Figure 5a, lane 4). This selective labeling indicated that the complementary tag−probe binding works even in a crude lysate including ATP or glutathione, competitive binder, or species reactive to the α-chloroacetyl Zn(II)-DpaTyr probe. Covalent labeling of CA6D4-MBP was further attempted inside *E. coli* cell. After incubation of *E. coli* cells overexpressing CA6D4-MBP with 20 μM of **3**-2Zn(II) in HBS buffer, confocal laser scanning microscopy revealed that **3**-2Zn(II) was localized inside the living cells as shown in Figure 5b. After 12 h at 4 °C, the cells were lysed and subjected to SDS-PAGE. A fluorescent band corresponding to the rhodamine-labeled CA6D4-MBP was predominant in the in-gel fluorescence analysis (Figure 5c, lane 3), whereas fluorescent bands were not detectable in the case of *E. coli* overexpressing C-MBP (Figure 5c, lane 4). These results indicated that selective protein labeling is possible even inside the *E. coli* cells, although the labeling efficiency was lower than in the protein mixture reported above.[10] We believe that the labeling efficiency in crude samples might be improved by enhanced binding affinity and selectivity of the tag−probe pair.

In conclusion, we have demonstrated the validity of combining selective molecular recognition with an organic reaction to achieve

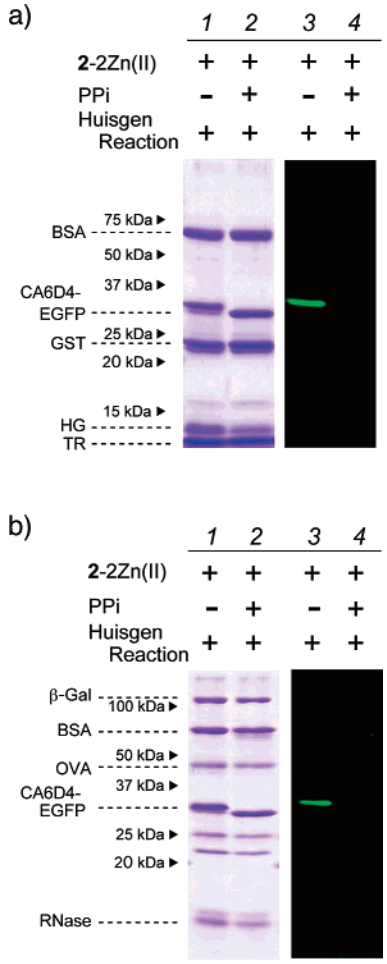

Figure 4. (a, b) SDS-PAGE analysis of the covalent labeling of CA6D4-EGFP with **2**-2Zn(II) in the protein mixture. The Coomassie staining (left) and in-gel fluorescence visualization (right) were performed after Huisgen reaction with coumarin azide **4**: BSA, bovine serum albumin; GST, glutathione *S*-transferase; HG, hemoglobin; TR, thioredoxin; β-Gal, β-galactosidase; OVA, ovalbumin; RNase, ribonuclease. Reaction conditions: 20 μM **2**-2Zn(II), 5 μM CA6D4-EGFP in 50 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.2, 20 °C.

site-specific covalent labeling of proteins. The present method using a genetically encodable reactive tag and the small molecular probe is potentially applicable to a wide variety of proteins, thus would provide a general tool for protein researches such as bioimaging, protein engineering, and biophysical studies of protein structure and dynamics.

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Supporting Information Available: Synthesis and characterization of the compounds, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

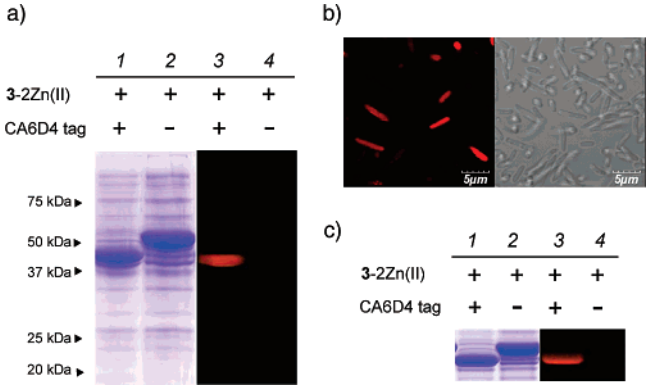

Figure 5. Covalent labeling of CA6D4-MBP or C−MBP in *E. coli* lysate (a) and inside *E. coli* cell (c) with **3**-2Zn(II). Reaction conditions: 20 μM **3**-2Zn(II) in HBS buffer, pH 7.4, 4 °C. (a, c) SDS-PAGE analysis of the labeling reaction in *E. coli* lysate overexpressing of CA6D4-MBP (lane 1 and 3) or C−MBP (lane 2 and 4). (b) confocal laser scanning (left) and differential interference contrast (DIC, right) images of *E. coli* expressing CA6D4-MBP incubated with **3**-2Zn(II).

References

(1) (a) O'Hare, H. M.; Johnsson, K.; Gautier, A. *Curr. Opin. Biol.* **2007**, *17*, 488. (b) Chen, I.; Ting, A. Y. *Curr. Opin. Biotechnol.* **2005**, *16*, 1.

(2) (a) Terpe, K. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 523. (b) Hauser, C. T.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3693. (c) Lata, S.; Gavutis, M.; Tampé, R.; Piehler, J. *J. Am. Chem. Soc.* **2006**, *128*, 2365. (d) Guignet, E. G.; Hovius, R.; Vogel, H. *Nat. Biotechnol.* **2004**, *22*, 440. (e) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269.

(3) (a) Gronemeyer, T.; Godin, G.; Johnsson, K. *Curr. Opin. Biotechnol.* **2005**, *16*, 453. (b) Carrico, I. S.; Carlson, B. L.; Bertozzi, C. R. *Nat. Chem. Biol.* **2007**, *3*, 321. (c) Los, G. V.; Wood, K. *Methods Mol. Biol.* **2006**, *356*, 195. (d) Lin, C.-W.; Ting, A. Y. *J. Am. Chem. Soc.* **2006**, *128*, 4542. (e) Chen, I.; Howarth, M.; Lin, W.; Ting, A. Y. *Nat. Methods* **2005**, *2*, 99. (f) George, N.; Pick, H.; Vogel, H.; Johnsson, N.; Johnsson, K. *J. Am. Chem. Soc.* **2004**, *126*, 8896. (g) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86.

(4) (a) Hwang, Y.; Thompson, P. R.; Wang, L.; Jiang, L.; Kelleher, N. L.; Cole, P. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 7621−7624. (b) Krusemark, C. J.; Belshaw, P. J. *Org. Biomol. Chem.* **2007**, *5*, 2201. (c) Scheck, R. A.; Francis, M. B. *ACS Chem. Biol.* **2007**, *2*, 247. (d) Evans, M. J.; Cravatt, B. F. *Chem. Rev.* **2006**, *106*, 3279. (e) Meredith, G. D.; Wu, H. Y.; Allbritton, N. L. *Bioconjugate Chem.* **2004**, *15*, 969.

(5) (a) Ojida, A.; Honda, K.; Shinmi, D.; Kiyonaka, S.; Mori, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2006**, *128*, 10452. (b) Honda, K.; Nakata, E.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2006**, 4024. (c) Honda, K.; Fujishima, S.; Ojida, A.; Hamachi, I. *ChemBioChem* **2007**, *8*, 1370.

(6) Powers, J. C.; Asgian, J.; Ekici, Ö. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639.

(7) The observed increase of the mass number 755 is smaller than the theoretical value 986 (**2**-2Zn(II)-HCl-3Cl). This shortage probably comes from the decomplexation of the Zn(II) ion(s) and/or the cleavage of the dipicolylamine (Dpa) fragment from the probe owing to the acidic pretreatment of the sample for desalting and harsh laser-induced ionization of the labeled protein in the MS analysis. The same shortage was observed in MALDI-TOF mass analysis of **1**-2Zn(II).

(8) Bräse, S.; Gil, C.; Knepper, K.; Zimmermann, V. *Angew. Chem., Int. Ed.* **2005**, *44*, 5188.

(9) The observed fluorescence only derives from the coumarin derivative **4** introduced into EGFP and not from EGFP. During the pretreatment for SDS-PAGE (95 °C, 3min), EGFP completely lost its fluorescence.

(10) The labeling reaction of **3**-2Zn(II) with the non overexpressed EGFP having the CA6D4 tag (<1% of total proteins) did not proceed in *E. coli* lysate, which is probably due to the competitive inhibition by phosphate derivatives such as ATP and the moderate binding affinity of the present tag−probe pair (K_d = 1.4 μM).

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