Site-specific covalent labeling of His-tag fused proteins with a reactive Ni(II)–NTA probe†

Sho-hei Uchinomiya,[a] Hiroshi Nonaka,[a] Sho-hei Fujishima,[a] Shinya Tsukiji,[a] Akio Ojida[ab] and Itaru Hamachi*[ac]

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A new method for covalent labeling of a His-tag fused protein with a small reactive probe was developed; this method is based on the complementary interaction between the His-tag and Ni(II)–NTA, which facilitates a nucleophilic reaction between a histidine residue of the tag and the electrophilic tosyl group of the Ni(II)–NTA probe by the proximity effect.

The complementary recognition pair of an oligo-histidine tag (His-tag) and a Ni(II) complex of nitrilotriacetic acid (Ni(II)–NTA), originally developed by the research group of F. Hoffmann-La Roche,[1] is now widely exploited as an indispensable tool for protein research. A wide variety of proteins are expressed as recombinants fused with a His-tag, by which affinity handling, purification and surface immobilization of proteins are largely facilitated.[2,3] In recent years, several groups reported an extension if the use of this pair for bio-imaging studies of His-tag fused proteins with a fluorescent Ni(II)–NTA probe, which further demonstrated its versatility as a tool in protein functional analysis.[4] These successful examples fully exploit the selective and strong binding properties of the His-tag–Ni(II)–NTA pair; however, the reversibility of this labeling technique sometimes limits its utility, particularly in post-labeling analyses such as SDS-PAGE or Western blotting. Development of an irreversible covalent labeling method should further extend the utility of the His-tag to various biochemical analyses.[5] Here, we report a new system for covalent protein labeling by using the His-tag–Ni(II)–NTA pair. The complementary interaction between the His-tag and the Ni(II)–NTA probe selectively facilitates a nucleophilic reaction between a histidine residue of the tag and the reactive benzenesulfonyl (*i.e.*, tosyl) group incorporated in the probe (Fig. 1).[6] This system enables us to site-specifically introduce diverse functional molecules into a His-tag site, allowing accurate post-labeling analysis and functional modification of the recombinant proteins.

The general design of the reactive Ni(II)–NTA probe and its reaction mode with a His-tag fused protein are illustrated in Fig. 1a. A tosyl unit was employed as a reactive linker between Ni(II)–NTA, a recognition site, and a functional group that is covalently attached to the His-tag. An advantageous point of this labeling system is that a His-tag fused protein is labeled with a small-sized functional unit, because the Ni(II)–NTA moiety is concomitantly released upon the substitution reaction. This is expected to minimize the problematic steric interference of introduced molecules to protein functions. We initially prepared a Ni(II)–NTA probe, **1**, bearing a fluorescent coumarin (Fig. 1) and evaluated its reactivity for the oligo-histidine peptides (Trp–Ala–$(His)_n$, n = 6 (His6) or 10 (His10)) in neutral aqueous solution. The MALDI-TOF mass analysis showed that the mono- and di-substituted peptides labeled with the coumarin unit of **1** were detected at m/e 1470 and 1688, respectively, after 7 h of reaction, indicating that the nucleophilic reaction of **1** with His6 peptide proceeded (Fig. 2a). To enhance the reactivity of the probe towards rapid labeling, we next prepared the halogen-substituted tosyl ester, **2** (Fig. 1), which should be more reactive owing to the electron-withdrawing effect of the substituted F and Cl. As the time trace plots of the labeling reactions monitored by MALDI-TOF (Fig. 2b) indicate, indeed, the reaction of **2** with His10 peptide proceeded most rapidly to yield the labeled product in over 40% yield after 7 h. The covalent adduct of **2**

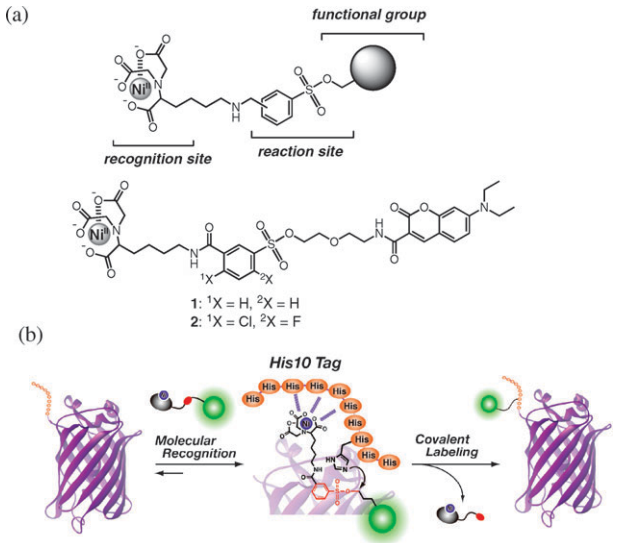

Fig. 1 (a) General design of the reactive Ni(II)–NTA probe (upper) and its fluorescent coumarin derivatives (lower). (b) Schematic illustration of the covalent modification of a His-tag fused protein using the reactive Ni(II)–NTA probe driven by the proximity effect.

[a] *Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Kyoto, 615-8510, Japan. E-mail: ihamachi@sbchem.kyoto-u.ac.jp*
[b] *JST, PRESTO (Life Phenomena and Measurement Analysis), Sanbancho, Chiyodaku, Tokyo, 102-0075, Japan*
[c] *JST, CREST (Creation of Next-generation Nanosystems through Process Integration), Sanbancho, Chiyodaku, Tokyo, 102-0075, Japan*

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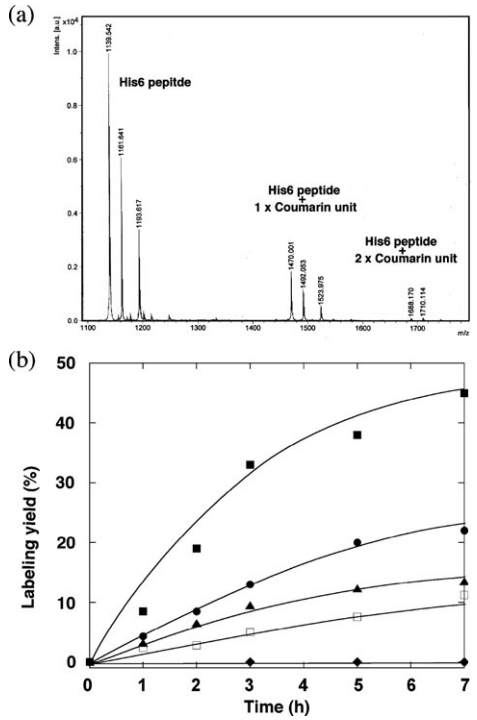

Fig. 2 (a) MALDI-TOF mass analysis of the covalent adduct of His6 peptide with **1**. *Reaction conditions:* 5 μM His6 peptide, 10 μM **1**, 50 mM HEPES, 100 mM NaCl, pH 7.2, 37 °C, 7 h. (b) Time-trace plot of the labeling reaction between His10 peptide and **2** (■), His10 peptide and **1** (●), His6 peptide and **2** (▲), His6 peptide and **1** (□), and *N*-Cbz-histidine and **2** (◆) based on the MALDI-TOF mass analysis. In the all reactions with the peptides, the mono-, and di-substituted peptides were detected in MALDI-TOF mass analysis.

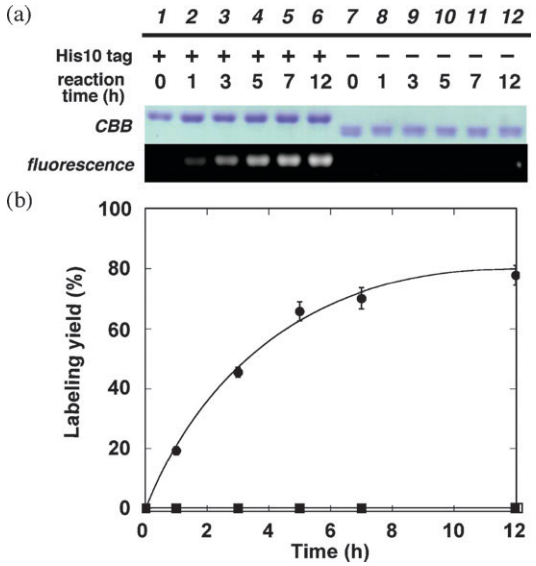

Fig. 3 (a) SDS-PAGE analysis of the labeling reaction of **2** with His10–EGFP (lanes 1–6) and control EGFP lacking a His10 tag (lanes 7–12). *Reaction conditions:* 5 μM His10–EGFP or control EGFP, 15 μM **2**, 50 mM HEPES, 100 mM NaCl, pH 7.2, 37 °C. (b) Time-trace plot of the labeling reaction of **2** with His10–EGFP (●) and with control EGFP (■) based on in-gel fluorescence analysis. The labeling yield (%) is defined as the amount of labeled coumarin units per total amount of protein, and calculates based on the fluorescent band intensity of the labeled protein relative to the authentic sample. The reaction was performed in triplicate and the error bars represent standard deviations.

was not detected in the reaction with a high concentration of a monomeric *N*-Cbz-histidine (400 μM) (Fig. 2b), suggesting that the reaction was effectively enhanced by the multivalent binding sites of the oligo-histidine peptides. Interestingly, the reaction with His10 peptide was significantly faster than with His6 peptide in both cases of **1** and **2**, probably due to the stronger binding affinity and/or the increased reaction probability of His10 peptide having more histidine residues than His6 peptide.[7]

The most reactive pair of **2** and His10-tag was subsequently applied to the covalent labeling of a His-tag fused protein. The labeling reaction was conducted between 15 μM **2** and 5 μM EGFP (enhanced green fluorescent protein), tethering the His10-tag at its *N*-terminal in neutral aqueous solution (50 mM HEPES, 100 mM NaCl, pH 7.2, 37 °C). Fig. 3a shows the fluorescence analysis of the labeling reaction by SDS-PAGE (in-gel fluorescence analysis), in which the fluorescent band of His10–EGFP, due to the labeled coumarin unit of **2**, gradually increased at the band of His10–EGFP (lanes 1–6), indicative of the progress of the covalent labeling reaction in a time-dependent manner. Fig. 3b shows a time-trace plot of the reaction estimated from the in-gel fluorescence analysis, showing that the labeling yield reached 80% after 12 h. In contrast, the covalent labeling did not occur with control EGFP lacking the His10-tag, even after 12 h (Fig. 3a, lanes 7–12, and Fig. 3b), suggesting that **2** effectively reacted at the His10-tag site of His10–EGFP by assistance of the coordination interaction with a μM range of dissociation constants (K_d, M).[7‡] To further confirm the labeling site of the protein, the labeled His10-tag fragment was selectively cleaved by thrombin digestion from His10–EGFP, and the EGFP unit was subjected to in-gel fluorescence analysis. Fluorescence was scarcely observed at the band of the cleaved EGFP unit in SDS-PAGE (ESI, Fig. S1†), whereas the mass peak assignable to the labeled peptide with one coumarin unit was detected in the cleaved His10 fragment by MALDI-TOF mass analysis (ESI, Fig. S2†). These results clearly indicate that the labeling reaction occurs at the His-tag site in a site-specific manner.

This covalent labeling system allows us to introduce a variety of functional groups into a His-tag fused protein. When the alkyne-tethered tosyl ester, **3**, was used as a reactive probe, the labeling reaction also proceeded to afford the His10–EGFP tethered to a terminal acetylene unit, which is a versatile reactive handle for Click chemistry.[8] Indeed, the labeling with **3**, followed by the subsequent Huisgen reaction with azide coumarin **4**, afforded the coumarin-appended EGFP, as shown by in-gel fluorescence analysis (Fig. 4a). We also employed the biotin-appended tosyl ester, **5**, for the reaction with His10–EGFP, in which the covalent attachment of the biotin unit was detected by chemical luminescence analysis using HRP (horseradish peroxidase)–avidin conjugate (Fig. 4b). These results demonstrated the general applicability of the present reactive tag system for protein labeling.

The specificity of this reactive tag system was evaluated in a labeling experiment using a crude lysate of *E. coli* cells expressing His10–EGFP. As shown in Fig. 5b, a single

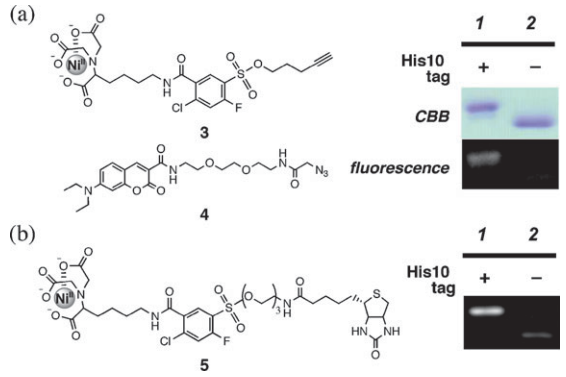

Fig. 4 Covalent labeling His10–EGFP with alkyne ester **3** (a) and biotin ester **5** (b). (a) In-gel fluorescence detection of the coumarin **4**-appended His10-EGFP, which was formed by the covalent protein labeling with **3** and the subsequent Huisgen reaction. (b) Chemical luminescence analysis of the biotin-appended His10–EGFP using HRP (horse radish peroxidase)–avidin conjugate. Lane 2 in (a) and (b) shows the experimental result using control EGFP lacking a His10 tag.

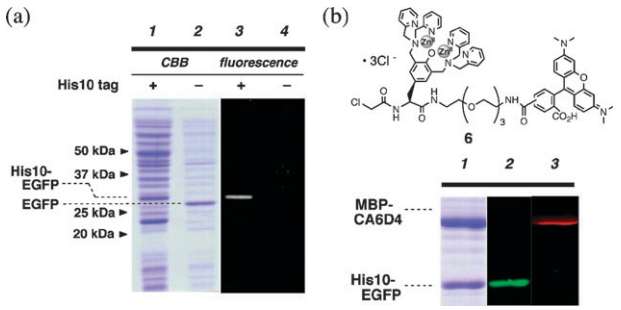

Fig. 5 (a) Selective covalent labeling of His10-EGFP with **2** in crude lysate of *E. coli* cells (lanes 1 and 2). Lanes 2 and 4 show the experimental result using EGFP lacking the His10 tag. (b) Orthogonal covalent labeling of His10–EGFP and CA6D4–MBP with the reactive probes **2** and **6** in crude lysate of *E. coli* cells. The detection channels of lanes 2 and 3 correspond to coumarin (480BP70) and rhodamine (630BP30) emission, respectively. Details of the experimental procedure are described in the experimental section in the ESI.†

fluorescence band corresponding to the coumarin-labeled His10–EGFP was observed (lane 3), whereas no fluorescence band was observed in the case of EGFP lacking His10-tag (lane 4) in in-gel fluorescence analysis. It is clear that the complementary recognition of the His-tag–Ni(II)–NTA pair and the subsequent selective labeling can work efficiently even under the complicated lysate conditions. The orthogonality against other protein labeling methods is another crucial point to assess the utility of this labeling system. As a reactive tag–probe pair, we recently developed a pair of Cys-tethered tetra-aspartate tags (CAAAAAADDDD: CA6D4-tag) and a binuclear Zn(II) complex (Zn(II)–DpaTyr) bearing a reactive chloroacetyl group.[9,10] Thus, the lysate sample of *E. coli* cells containing His10-fused EGFP and CA6D4-fused MBP (maltose binding protein) was subjected to one-pot labeling experiments using the corresponding fluorescent probes **2** and **6**, respectively. Fig. 5b clearly shows that His10-fused EGFP is exclusively labeled with **2** but not with **6**, and *vice versa*, that is, CA6D4-fused MBP is labeled with **6** but not with **2**. It is remarkable that these two reactive tag systems are orthogonal to each other in the covalent protein labeling, in spite of the fact that similar nucleophilic reactions occur in both methods. This sufficient orthogonality can be mainly ascribed to the high recognition selectivity of each His10–Ni(II)–NTA and D4–Zn(II)–DpaTyr pair.[10]

In summary, we have developed a new His-tag based reactive tag–probe pair for site-specific covalent modification of proteins, on the basis of our originally developed protein labeling method using a reactive tosyl chemistry.[6] This proximity-driven covalent labeling method should be useful as a new tool for post-labeling analysis, functional modification, as well as manipulation of proteins of interest, which cannot be readily achieved by the conventional His-tag–Ni(II)–NTA pair that relies on non-covalent interactions. We have also demonstrated that the present labeling method is orthogonal to the other reactive tag–probe pair of CA6D4-tag–Zn(II)–DpaTyr. This makes us envision that the combinational use of these orthogonal labeling systems would be a promising strategy to elucidate complicated biological events involving multiple proteins such as the formation or de-formation of protein signaling complexes. We believe that the present non-enzymatic labeling method would be useful for bio-imaging studies as a complementary tool to the existing enzyme-catalyzed protein labeling methods,[11] though further improvements might be needed for such living cell applications. Our research is now ongoing along these lines.

Notes and references

‡ The labeling yield dramatically decreased to 11% (after 7 h) when the reaction was carried out in the absence of Ni(II) ions. This result also suggests that the coordination interaction between the Ni(II)–NTA site of **2** and the His10-tag works effectively to enhance the reaction.

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