Ratiometric fluorescence detection of a tag fused protein using the dual-emission artificial molecular probe†

Kei Honda, Eiji Nakata, Akio Ojida and Itaru Hamachi*

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We have successfully developed a ratiometric detection system for protein of interest using the complementary recognition pair of the tetra-aspartate peptide tag and the SNARF-appended Zn(II)–DpaTyr probe.

The sensing and imaging of a protein of interest is of great importance for the understanding of protein functions in complicated biological events.[1] Unlike the conventional protein labeling method using antibodies or the genetic fusion of a fluorescent protein such as green fluorescent proteins (GFPs),[2] a pair of peptide tags fused to a protein and a complementary fluorescent binding probe is now being recognized as a promising labeling technique.[3] Thus, several tag–probe pairs have been actively developed.[4] In protein labeling using an artificial probe, it is highly desirable to couple the binding event of the probe with a fluorescence change in its intensity or wavelength, because such a change significantly contributes to reduce the background signal due to the unbound probe, thereby facilitating precise detection or imaging of the target protein even under complicated biological conditions.[5] Although Tsien and coworkers reported an exclusive pair of tetra-cysteine tag–FlAsH probe that showed a large fluorescence enhancement in the labeling process,[6] useful pairs that exhibit a ratiometric emission change have been poorly developed,[7] irrespective of its versatility for quantitative analysis in bio-imaging experiments.

We recently proposed a peptide tag–artificial probe pair as a new protein labeling system.[8] This system is composed of the sequential tetra-aspartate tag (D4-tag) and the fluorescent binuclear Zn(II) complex (Zn(II)–DpaTyr) as a complementary binding pair, being applicable to labeling a cell surface membrane protein. In this communication, we describe that a fluorescence ratiometric detection mode is successfully equipped to the D4-tag–Zn(II)–DpaTyr system by utilizing the binding-induced micro pH change (Fig. 1). The ability of this ratiometric sensing was clearly demonstrated by the fluorescence detection of D4-tagged RNase in a homogeneous neutral aqueous solution.

It seems reasonable to assume that the local pH in the proximity to the D4-tag is rather acidic than the bulk pH due to its accumulated carboxylate residues. On the basis of this assumption, we designed a new probe **1**–2Zn(II), which possesses a pH-responsive dual-emission fluorophore as a sensory unit. Among various fluorophores, seminaphthorhodafluor (SNARF),[9] a representative

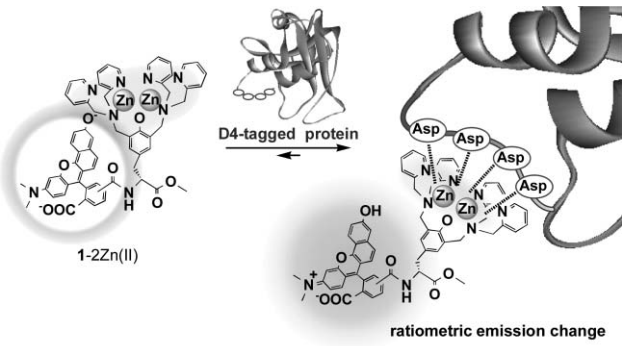
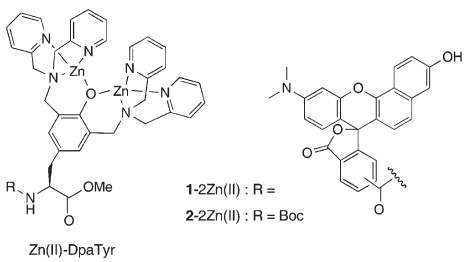


Fig. 1 New ratiometric fluorescence detection system of the D4-tagged protein.

pH indicator that shows a dual-emission change in the neutral pH region, was appended to the Zn(II)–DpaTyr. **1**–2Zn(II) was synthesized by the simple condensation reaction of the DpaTyr unit with a carboxy SNARF, followed by Zn(II) complexation with two equivalents of $ZnCl_2$ (Scheme S1†). The compound characterizations of the SNARF ligand **1** and **1**–2Zn(II) were performed by ^{1}H-NMR and high resolution mass spectroscopy.



Fluorescence titration of **1**–2Zn(II) with a D4-tag model peptide (D4: Boc-DDDD-NH_2) was conducted, prior to application of the present system to proteins. Fig. 2 showed the fluorescence spectral change of **1**–2Zn(II) upon addition of D4 peptide under neutral aqueous conditions (50 mM HEPES, pH 7.2). Typical ratiometric fluorescence change immediately occured in response to D4 peptide addition (within 5 s), in which the emission at 628 nm due to the basic phenolate form of the SNARF decreased and the emission at 586 nm due to its acidic phenol form concurrently increased with an emission isosbestic point at 610 nm. The plot of the emission intensity ratio R (586 nm/628 nm) showed a good saturation behavior (ΔR = 0.57), and the curve-fitting analysis afforded the binding constant of $1.1 \pm 0.1 \times 10^6$ M^{-1} (Fig. S1†). This value is in good agreement with that of the **2**–2Zn(II) (*i.e.*, without SNARF unit) with D4 peptide determined by ITC

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Nishikyo-ku, Kyoto, 615-8510, Japan. E-mail: ihamachi@sbchem.kyoto-u.ac.jp; Fax: +81-75-383-2759; Tel: +81-75-383-2757

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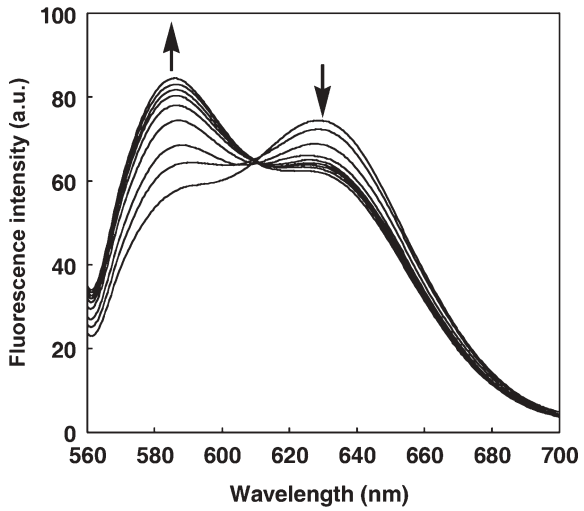

Fig. 2 Fluorescence spectral change of **1**–2Zn(II) (2 μM) upon addition of D4 peptide: [D4 peptide] = 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 14 μM in 50 mM HEPES buffer, pH 7.2, at 25 °C, λ_{ex} = 543 nm (isosbestic point).

experiment (K_{app} = 6.9 ± 0.2 × 10^5 M^{-1}) or induced circular dichroism (*i*-CD) titration experiment (K_{app} = 1.4 ± 0.2 × 10^6 M^{-1}).[8] On the other hand, no significant spectral change was observed when a carboxy SNARF dye (*i.e.*, without Zn(II)–DpaTyr unit) was titrated with D4 peptide (data not shown). These results indicate that the binding of **1**–2Zn(II) with D4 peptide can be read-out by the ratiometric emission change.

In order to clarify the mechanism of the ratiometric emission change, we next evaluated the pK_a value of the SNARF unit attached to **1**–2Zn(II) in the absence or presence of D4 peptide. From the pH-dependent absorption spectral change of **1**–2Zn(II) (inset of Fig. 3), the pK_a of the SNARF unit in the absence of D4 peptide was determined to be 6.6 (Fig. 3), which is one order of magnitude lower than the carboxy SNARF dye (pK_a = 7.6). This pK_a shift suggests that the basic phenolate form of the SNARF unit is stabilized by the adjacent cationic Zn(II)–Dpa unit. Interestingly, this stabilization effect was canceled in the presence of D4 peptide (Fig. S2†) and in fact, the pK_a value of the SNARF unit of **1**–2Zn(II) was restored to that of the original SNARF (pK_a = 7.5) (Fig. 3). These results clearly indicate that the ratiometric emission change induced by the binding with D4 peptide can be ascribed to the pK_a shift of the SNARF unit as a result of the electrostatic neutralization of the cationic **1**–2Zn(II).

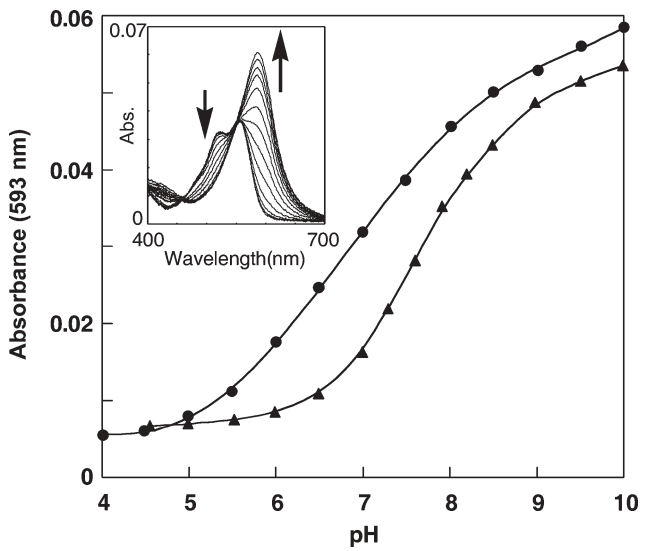

Fig. 3 The pH-dependent absorbance change (detected at 593 nm) of **1**–2Zn(II) (2 μM) in the absence (closed circles) and presence (closed triangles) of D4 peptide (20 μM) in 10 mM MES/HEPES buffer at 25 °C. (inset) Absorption spectral change of **1**–2Zn(II) (2 μM) at various pH conditions (from pH 4.0 to pH 10.0) in 10 mM MES/HEPES buffer at 25 °C.

Subsequently, we applied the present ratiometric sensing system to the D4-tag fusion protein. As a proof-of-principle, ribonuclease (RNase) tethered to a D4-tag at its *N*-terminus (D4–RNase) was employed. When D4–RNase was added to an aqueous solution of **1**–2Zn(II) (2 μM in 50 mM HEPES buffer, pH 7.2), a seesaw type of the dual-emission change similar to the case of D4 peptide was immediately observed. The plot of the emission intensity ratio *R* (586 nm/628 nm) displayed good saturation behavior (Fig. 4), giving the binding constant to be 8.2 ± 5.4 × 10^5 M^{-1}, the value of which is in good agreement with that of the D4 peptide. In contrast, the emission ratio scarcely changed in the case of the native RNase or His_6-tag (HHHHHH) tethered RNase (Fig. 4), because of the lack of significant interactions of **1**–2Zn(II) with a specific protein surface.[8] These results clearly indicate that the ratiometric detection mode incorporated into the D4-tag–**1**–2Zn(II) pair can effectively operate on a protein surface under homogeneous, neutral, aqueous conditions.

In conclusion, we have developed a fluorescence ratiometric detection system of a D4-tagged protein using the SNARF-appended Zn(II)–DpaTyr. The mechanism of the dual emission sensing is attributable to the pK_a shift of the SNARF unit of **1**–2Zn(II) induced by binding to the D4-tag. We envision further applications of this ratiometric detection system not only for quantitative detection of a tagged protein in the test tube, but also for protein labeling on cell surface membranes.

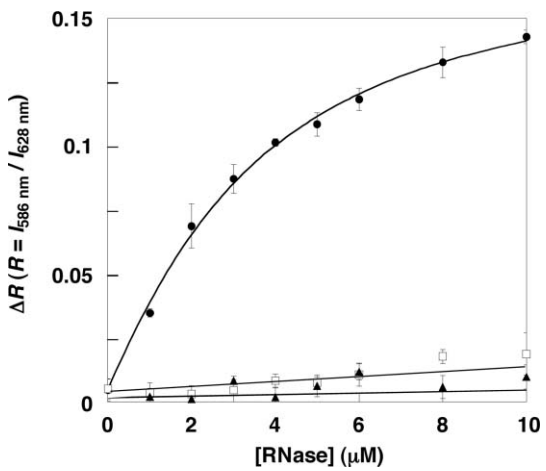

Fig. 4 The changes of the fluorescence intensity ratio *R* ($R = I_{586nm}/I_{628nm}$) of **1**–2Zn(II) (2 μM) upon addition of D4–RNase (closed circles), His_6–RNase (closed triangles) or native RNase (opened squares) in 50 mM HEPES buffer, pH 7.2, at 25 °C, λ_{ex} = 543 nm.

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