Sequence selective dual-emission detection of (*i*, *i* + 1) bis-phosphorylated peptide using diazastilbene-type Zn(II)-Dpa chemosensor†‡

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This paper describes a new fluorescent chemosensor for phosphorylated peptide, which comprises a rigid *trans*-4,4′-diazastilbene and two Zn(II)-Dpa (2,2′-dipicolylamine) units; this chemosensor sequence-selectively binds to a (*i*, *i* + 1) bis-phosphorylated peptide and displays a dual-emission fluorescence change.

Protein phosphorylation is a prevalent post-translational modification crucial for controlling many protein functions. A variety of biological and chemical techniques have been recently developed to realize precise and sensitive detection of these phosphorylation events.[1,2] Among them, fluorescence sensing with a small molecular-based chemosensor is a promising technique to directly detect protein phosphorylation in a convenient manner without the need for special instruments. Several fluorescent chemosensors have been recently developed for this purpose, which displayed a fluorescent signal change upon binding to phosphorylated proteins and peptides.[3–7] Although they might be useful to simply detect whether protein is in a phosphorylated state or not,[8–10] sequence- or site-specific detection of a certain protein phosphorylation among various phosphorylation sites still remains a challenge.

We have recently reported that binuclear Zn(II)-Dpa (2,2′-dipicolylamine) complexes such as **2**-2Zn(II) strongly bind to bis-phosphorylated peptides in neutral aqueous conditions through cross-linking interaction with the two phosphorylated residues (Fig. 1).[5,6] Although **2**-2Zn(II) showed a high binding selectivity for bis-phosphorylated peptides (maximally 10^7 M^{-1} in binding affinity) against mono-phosphorylated ones, the binding selectivity among the doubly phosphorylated peptides at the different (*i*, *i* + *n*) positions (*n* = 4, 8, 12) was moderate. This is partly due to its rather flexible structure, which allows the conformation change to result in non-selective binding with these peptides. Another drawback of **2**-2Zn(II) is its small fluorescence signal change at a relatively shorter wavelength upon binding to a bis-phosphorylated peptide, which makes it difficult to precisely analyze a certain protein phosphorylation event. In this communication, we report a new binuclear Zn(II)-Dpa complex **1**-2Zn(II) as a fluorescent chemosensor for bis-phosphorylated peptide. The unique molecular design of **1**-2Zn(II), in which the Zn(II)-Dpa sites and the fluorescent unit are directly conjugated with a rigid *trans*-4,4′-diazastilbene unit, provides a high binding selectivity for (*i*, *i* + 1) bis-phosphorylated peptide and a clear dual-emission signal change in the formation of the binding complex.

The synthesis of **1**-2Zn(II) is outlined in Scheme 1. Briefly, vinylpyridine derivative **4** was obtained by Stille coupling, followed by an olefin metathesis reaction using Grubb's catalyst to yield *trans*-4,4′-diazastilbene **5**. Acid catalyzed deprotection and subsequent reductive amination with *N*-methylpicolylamine afforded the ligand **1**. Finally, complexation with 2 equiv. of $Zn(NO_3)_2$ yielded the **1**-2Zn(II). Details of the synthetic procedure for **1**-2Zn(II) are provided in the ESI.‡

The absorption maximum of **1**-2Zn(II) is observed at 294 nm, and when the complex is excited at 294 nm, the emission maximum is 385 nm (Fig. 2). The fluorescence quantum yield (Φ) of **1**-2Zn(II) is below 0.01 under aqueous neutral conditions (50 mM HEPES buffer, pH 7.2). To evaluate the sensing selectivity of **1**-2Zn(II) for bis-phosphorylated peptides, we performed the fluorescence titration with a series of phosphorylated peptides possessing two phosphorylated amino acid residues at (*i*, *i* + *n*) positions (*n* = 1, 2, 3, 4, 6), all of which are derived from the natural sequence of the hyperphosphorylated tau protein reported in the literature (Table 1).[11] Fig. 2 shows the fluorescence spectral change of **1**-2Zn(II) upon addition of Tau(400–409)-2P(*i* + 1) peptide. Interestingly, a clear dual-emission change took place, that is the initial emission maximum at 385 nm gradually decreased

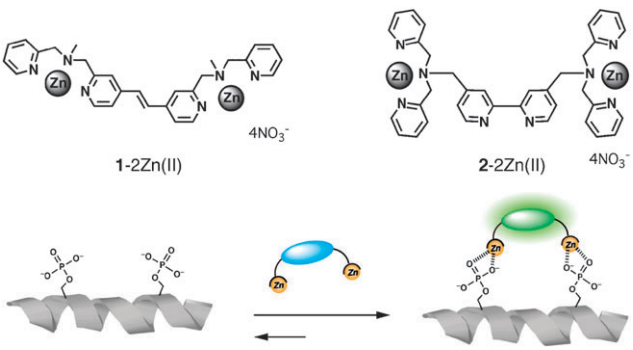

Fig. 1 Structures of fluorescence probes for bis-phosphorylated peptides (above), and illustration of their cross-linking binding with bis-phosphorylated peptide (below).

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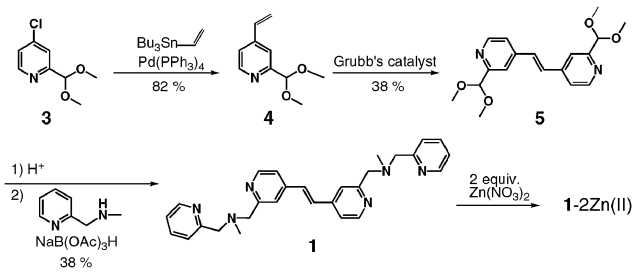

Scheme 1 Synthesis of **1**-2Zn(II).

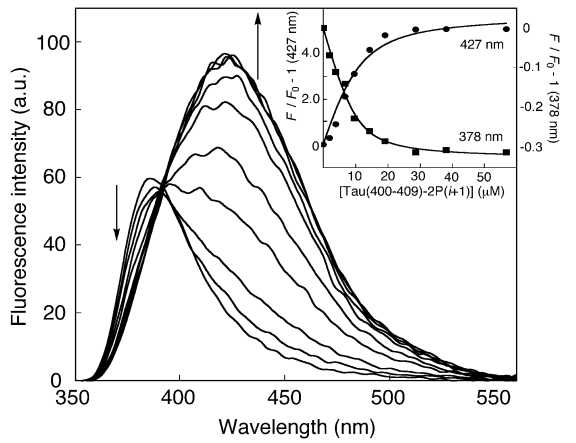

Fig. 2 Fluorescence spectral changes of **1**-2Zn(II) (10 μM) in 50 mM HEPES buffer at pH 7.2 upon addition of Tau(400–409)-2P(i + 1) at 25 °C, λ_{ex} = 323 nm. Inset: Fluorescence titration profile of **1**-2Zn(II) (λ_{em} = 378 nm and 427 nm) against the concentration of Tau(400–409)-2P(i + 1). The solid line is the best fit of the data to the equation for 1 : 1 complex formation.

and a new emission at a longer wavelength (λ_{max} = 425 nm) concomitantly emerged. This signal change allowed us to detect 10^{-6} M of the peptide in the fluorescence titration. The ratio plot of the fluorescence intensity at two wavelengths (378 nm and 427 nm) shows that the ratio value R increases up to nearly 4-fold (Fig. 4). The binding constant of **1**-2Zn(II) with Tau(400–409)-2P(i + 1) peptide was evaluated to be 6.1×10^5 M^{-1} by curve fitting analysis. This binding behavior was further evaluated by ITC (isothermal titration calorimetry, Fig. 3), showing a binding constant (K_a) of 6.3×10^5 M^{-1} with a 1 : 1 stoichiometry (N = 1.02), almost identical with data from the fluorescence titration. It is clear that the emission response of **1**-2Zn(II) directly reflects the binding to

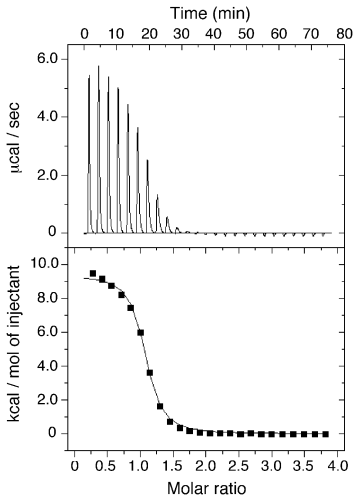

Fig. 3 Isothermal calorimetric titration of **1**-2Zn(II) (0.1 mM) with 25 aliquots (10 μL each) of Tau(400–409)-2P(i + 1) (2.0 mM) in 50 mM HEPES buffer at pH 7.2 and 25 °C. The solid line represents the best fit of the experimental data.

the bis-phosphorylated peptide. None of the fluorescence change was induced by the corresponding mono-phosphorylated peptide Tau(400–409)-1P (Table 1), implying that **1**-2Zn(II) interacts with the bis-phosphorylated Tau(400–409)-2P(i + 1) peptide through the cross-linking binding mode so as to show a dual-emission change with a strong binding affinity. CD measurement of the mixture of **1**-2Zn(II) and Tau(400–409)-2P(i + 1) showed a large Cotton peak in the absorbance region of **1**-2Zn(II) from 230 to 380 nm (Fig. S1‡), further supporting the formation of a conformationally restricted cross-linking binding complex. The dual-emission change was also observed in the titration with the (i, i + 2) bis-phosphorylated peptides such as Tau(204–217)-2P(i + 2) and Tau(231–238)-2P(i + 2), however the emission changes (Fig. 4) and the binding affinities (Table 1) were apparently smaller than in the case of Tau(400–409)-2P(i + 1) peptide. The fluorescence changes and the binding affinities were also small for other peptides that have the two phosphorylated residues at more distant positions, such as Tau(210–220)-2P(i + 3), Tau(204–217)-2P(i + 4), and Tau(204–217)-2P(i + 6) (Fig. 4 and Table 1). Overall these results indicate that **1**-2Zn(II) is a selective chemosensor for (i, i + 1) bis-phosphorylated peptide with a dual-emission signal change. This high sequence selectivity would be reasonably ascribed to

Table 1 Binding constants for the complexation of **1**-2Zn(II) with phosphorylated peptide determined by a fluorescence titration experiment

Peptide sequence[a]		P-sites[b]	10^5 K_{app}/M^{-1}[c,d]
Tau(400–409)-1P :	YSGDT**pS**PRHLS	i	—[e]
Tau(400–409)-2P(i + 1) :	YSGD**pT**P**pS**PRHLS	i, i + 1	6.1 (6.3)
Tau(204–217)-2P(i + 2) :	YGTPGSRSR**pT**P**pS**LPT	i, i + 2	0.2 (0.8)
Tau(231–238)-2P(i + 2) :	YTPPK**pS**P**pS**S	i, i + 2	0.6
Tau(210–220)-2P(i + 3) :	YSRTP**pS**LP**pT**PPT	i, i + 3	0.2
Tau(204–217)-2P(i + 4) :	YGTPG**pS**RSR**pT**PSLPT	i, i + 4	0.08
Tau(204–217)-2P(i + 6) :	YGTPG**pS**RSRTP**pS**LPT	i, i + 6	—[e]

[a] N-terminal tyrosine (Y) was introduced for determination of the peptide concentration from UV absorbance. [b] P-sites designates the position of phosphorylated amino acid residues on the peptide. [c] Binding constants (K_{app}) were determined by curve fitting with a theoretical equation for 1 : 1 complexation. [d] The values in parentheses were determined by an ITC experiment. [e] Binding constant could not be evaluated due to the small increase of the fluorescence intensity (λ_{em} = 427 nm).

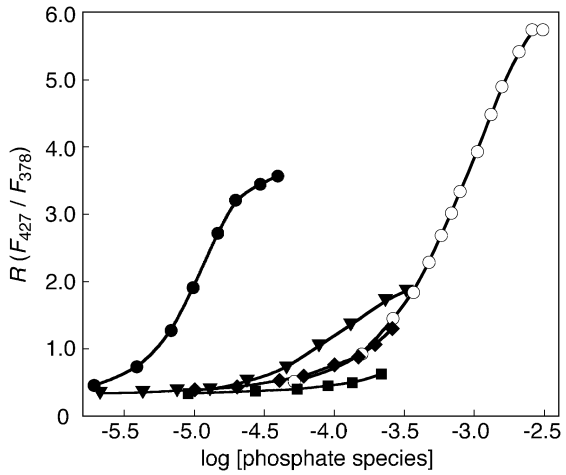

Fig. 4 Changes of the ratio value R (F_{427}/F_{378}) of **1**-2Zn(II) (10 μM in 50 mM HEPES buffer at pH 7.2) in the titration with Tau(400–409)-2P(i + 1) (●), Tau(204–217)-2P(i + 2) (▼), Tau(210–220)-2P(i + 3) (◆), Tau(204–217)-2P(i + 4) (■) and inorganic phosphate (○).

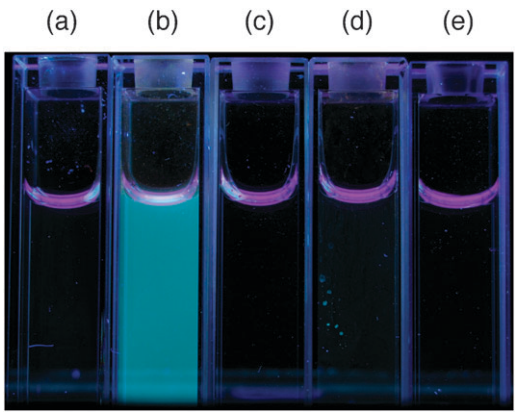

Fig. 5 Photograph of the aqueous solutions of **1**-2Zn(II) (20 μM) in the absence (a) and presence of the phosphorylated peptide (40 μM): Tau(400–409)-2P(i + 1) (b), Tau(204–217)-2P(i + 2) (c), Tau(210–220)-2P(i + 3) (d), Tau(204–217)-2P(i + 4) (e) irradiated with 365 nm light.

the rigid and compact structure of **1**-2Zn(II), which is suitable to bind to the two phosphate groups positioned adjacent to each other.

Fig. 5 displays a photograph of the aqueous solution of **1**-2Zn(II) in the absence and presence of various bis-phosphorylated peptides. Distinct blue–green fluorescence was selectively observed in the solution containing Tau(400–409)-2P(i + 1) peptide, whereas the blue emission was negligible in the case of Tau(204–217)-2P(i + 2), Tau(210–220)-2P(i + 3) and Tau(204–217)-2P(i + 4). Thus, we can visually discriminate the (i, i + 1) peptide from other bis-phosphorylated peptides by using **1**-2Zn(II) as a fluorescent indicator.

To gain insight into the mechanism of the dual-emission change, we performed several spectroscopic experiments. Almost the same excitation spectra were obtained from the solution of **1**-2Zn(II) (λ_{em} = 385 nm) and its mixture with Tau(400–409)-2P(i + 1) (λ_{em} = 427 nm), indicating that the emission shift is caused by the electronic configuration change in the excitation state. Also, we noticed that an almost identical spectral change occurred on addition of inorganic phosphate although the affinity is much smaller than for the (i, i + 1) peptide (See Fig. 4).§ This implies that the cross-linking binding which may affect the planarity of the diazastilbene fluorophore is not essential for the dual-emission changes.[12]¶ Instead, it may be proposed that the coordination of phosphate anions to Zn(II)-Dpa sites modulates an excited-state through several factors such as a solvent reorientation process, resulting in the emission shift. Further photophysical study is required to fully understand the detailed sensing mechanism.

In summary, we have revealed that **1**-2Zn(II) is a selective fluorescent chemosensor for (i, i + 1) bis-phosphorylated peptide with a dual-emission change among various phosphorylated peptides. This sensing selectivity and signal change are achieved on the basis of the simple but sophisticated molecular design of **1**-2Zn(II), *i.e.*, direct conjugation of the Zn(II)-Dpa sites to the rigid fluorophore with a minimized conformational flexibility. As a general design strategy, extension of the conjugated olefin unit of the chemosensor may allow us to create fluorescent chemosensors selective for other types of peptides doubly phosphorylated at more distant positions. Our research is ongoing along this line.

Notes and references

§ **1**-2Zn(II) also showed a similar dual-emission change towards inorganic pyrophosphate (PPi) and inositol 1,4,5-triphosphate (IP_3) with a strong binding affinity ($K_{app} > 10^6$ M^{-1}), suggesting that these poly-phosphate species competitively inhibit the sensing of the phosphorylated peptides.

¶ Little emission shift was observed in the temperature-dependent fluorescence measurement from 25 to −78 °C, also suggesting that the emission shift is not simply caused by the conformational rigidification of the diazastilbene unit in the cross-linking binding.

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