J|A|C|S
COMMUNICATIONS

Published on Web 05/09/2002

First Artificial Receptors and Chemosensors toward Phosphorylated Peptide in Aqueous Solution

Akio Ojida,[†] Yasuko Mito-oka,[†] Masa-aki Inoue,[†] and Itaru Hamachi*[,†,‡,§]

PRESTO (Organization and Function, JST), Institute for Fundamental Research of Organic Chemistry (IFOC), Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka, 812-8581, Japan

Received January 30, 2002

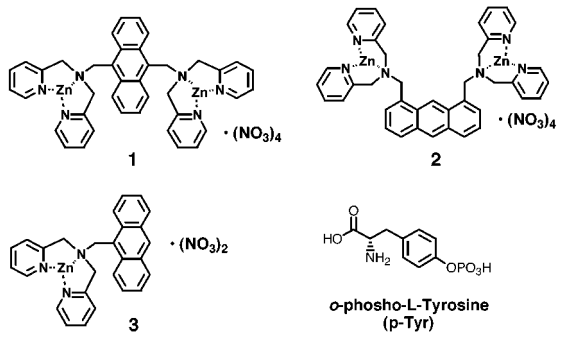
We describe herein novel fluorescent chemosensors toward a phosphorylated peptide surface. Anthracene derivatives having two sets of zinc(II)-dipicolylamine selectively bind phosphorylated chemical species so as to cause the fluorescent spectral change. It is clear that these chemosensors can detect a consensus peptide sequence phosphorylated by v-Src with the high affinity (10^7 M^{-1}) in aqueous solution.

Posttranslational modification of native proteins is essentially important for their functions in many biological systems. Especially, phosphorylation, dephosphorylation, or both which occur site-specifically on a protein surface are widely involved in switching the enzymatic activity and consequently play one of the key roles in signal transduction cascades of living cells.[1] To understand such complicated signal transduction schemes in molecular terms, it is strongly desirable to develop versatile methods and molecular probes which can selectively recognize phosphorylated proteins and enzymes with convenient means.

Although fluorescent chemosensor is one of the ideal systems,[2] there are few examples of phosphate anion sensors that can be used in aqueous solution.[3] In a pioneering work, Czarnik and co-workers reported that a polyamine-appended anthracene acts as a fluorescent phosphate sensor in aqueous solution.[4] The affinity constant, however, is not high enough to apply this sensor to the phosphorylated peptides or proteins. Among naturally occurring phosphatases, on the other hand, coordination chemistry provided by metal complexes is often employed to bind a phosphate unit of substrates in the enzymatic pocket.[5] This gave us an important clue to design an appropriate binding motif toward the phosphate unit.[6] Therefore, we decided to utilize dipicolylamine zinc(II) complex for the phosphate binding as an initial attempt (Chart 1). Corresponding halomethylanthracene derivatives reacted with dipicolylamine, followed by zinc(II) complexation to afford the artificial receptors **1**, **2**, and **3**.[7]

When phosphorylated tyrosine (p-Tyr) was added to the aqueous solution of the dinuclear **1**, we found the remarkable increase of fluorescence due to the anthracene moiety (Figure 1). In contrast, addition of tyrosine (Tyr) did not cause any fluorescent change. The dinuclear receptor **2** displays the fluorescent increase by p-Tyr similar to **1**, whereas such a change is not observed for the mononuclear receptor **3**. Next, the fluorescence titration with various anionic species was conducted to examine the anion selectivity of the receptors **1** and **2**. Figure 2 shows semilog plots of their emission intensity changes depending on the anion concentration. It is clear that these receptors strongly bind and fluorometrically sense

Chart 1



phosphorylated species such as phosphate and p-Tyr in the range of 10^{-6}−10^{-5} M in a neutral aqueous solution. Among the other anions, addition of bicarbonate gradually intensified the fluorescence in the 10^{-3} M range of concentration, and azide anion lessened the fluorescence intensity in the same concentration range. No fluorescent response is induced by other anions such as sulfonate, nitrate, acetate, and chloride, revealing that dinuclear receptors **1** and **2** show the high selectivity toward phosphate derivatives. Job's plot fluorescently examined in the binding experiments of these receptor and p-Tyr shows 1:1 stoichiometry (inset of Figure 2a).

The NMR study affords the structural information of the 1:1 complex. Upon the complexation of **2** with p-Tyr, the proton signals of the pyridine ring of two Dpa slightly shifted to the upfield (0.09−0.18 ppm) and the corresponding proton signals are not distinguishable among the four pyridine rings. This observation suggests that two zinc-Dpa sites equally contribute to the binding to the phosphate site. Such a cooperative action may reasonably explain the high affinity of the present dinuclear receptors toward p-Tyr even in aqueous solution. Two sets of three methylene protons connected to the tertiary nitrogen (4.63, 4.29, 3.65 ppm) are also indistinguishable from each other and become sharper upon the p-Tyr binding. This indicates the complexation-induced conformational rigidification of the receptor, which can attribute to the increase of the fluorescence quantum yield. In the case of p-Tyr, the hyrdophobic microenvironment provided by the aromatic ring is an additional cause of the fluorescent intensification.[8]

On the basis of the above results, we subsequently examined the sensing ability of these receptors toward phosphorylated peptides in aqueous solution. Three sorts of phosphorylated peptides (peptide-a, -b, -c) which are consensus sequences modified by distinct kinases were used, together with a nonphosphorylated peptide (peptide-d, See Chart 2).[9] The fluorescence intensity of **1** significantly increases by addition of less than 1 μM of peptide-a, a consensus Glu-rich peptide sequence phosphorylated by v-Src,[10,11] as shown in Figure 3a. Along with the great increase of the

* To whom correspondence should be addressed. E-mail: itarutcm@mbox.nc.kyushu-u.ac.jp.
† Kyushu University.
‡ PRESTO.
§ Institute for Fundamental Research of Organic Chemistry (IFOC).

10.1021/ja025761b CCC: $22.00

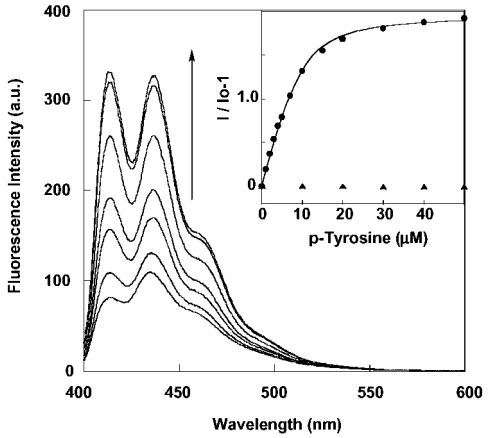


Figure 1. Fluorescence spectral change of **1** (10 μM) upon the addition of *o*-phospho L-tyrosine (p-Tyr): [p-Tyr] = 0, 1.0, 3.0, 5.0, 10, 30, 50 μM from the lowest to the top trace. The spectra were measured in 10 mM HEPES buffer (pH 7.2) at 20 °C, λ_{ex} = 380 nm. (Inset) Fluorescent titration curve of **1** (●) and **3** (▲) with p-Tyr.

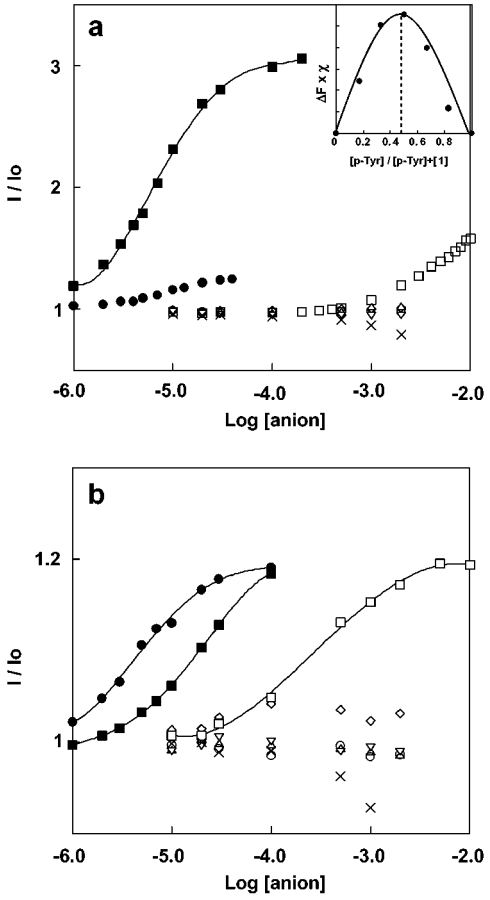


Figure 2. Relative fluorescence emission response of (a) **1** and (b) **2** to the anion concentration (log[anion]): phosphate (●), p-Tyr (■), chloride (○), acetate (◇), sulfate (△), nitrate (▽), carbonate (□), azide (×). The spectra were measured in 10 mM HEPES buffer (pH 7.2) at 20 °C. λ_{ex} (**1**) = 380 nm, λ_{ex} (**2**) = 370 nm. Inset: the Job's plot fluorescently examined between **1** and p-Tyr.

fluorescence (4- or 5-fold magnitude), the binding is remarkably strong with the affinity of 10^7 M^{-1}. In contrast, the fluorescence change was scarcely observed for the nonphosphorylated peptide-d up to the more than 10 μM, revealing that the receptor **1** can distinguish the phosphorylated peptide from the nonphosphorylated one. The fluorescent titration data for all peptides are summarized

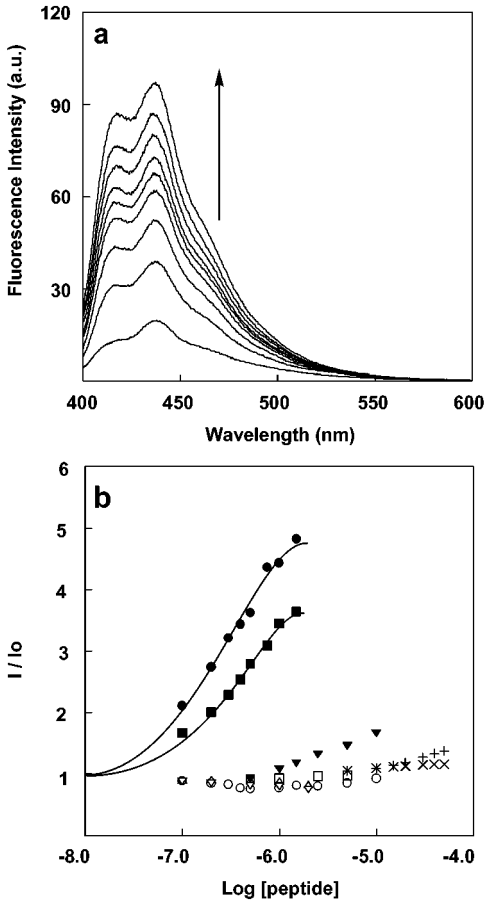
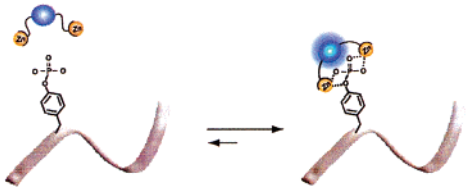


Figure 3. (a) Fluorescence spectral change of **1** (0.5 μM) upon the addition of peptide-a: [peptide-a] = 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5 μM in 50 mM HEPES buffer (pH 7.2) at 20 °C, λ_{ex} = 380 nm. (b) Fluorescence titration profiles of **1** and **2** with the peptide a−d: **1** with peptide-a (●), peptide-b (△), peptide-c (+), peptide-d (○), **2** with peptide-a (■), peptide-b (▽), peptide-c (×), peptide-d (□), and **3** with peptide-a (▼). The detailed experimental conditions are described in Supporting Information.

Chart 2. (Top) Amino Acid Sequences of the Phosphorylated Peptides and (Bottom) Schematic Representation of the Interaction of Receptor **1**, **2** with a Phosphorylated Peptide

peptide a: Glu-Glu-Glu-Ile-pTyr-Glu-Glu-Phe-Asp
peptide b: Arg-Arg-Phe-Gly-pSer-Ile-Arg-Arg-Phe
peptide c: Lys-Ser-Gly-pTyr-Leu-Ser-Ser-Glu
peptide d: Glu-Glu-Glu-Ile-Tyr-Glu-Glu-Phe-Asp



in Figure 3b and Table 1. Among three phosphorylated peptides, peptide-b, a consensus Arg-rich peptide sequence phosphorylated by Bck-1,[10] is not sensed at all, and peptide-c, EGFR phosphorylating domain of ezrin,[11] is moderately recognized by **1** at >10 μM level. Since the dinuclear Zn(Dpa) receptor bears positive charges, the electrostatic attraction is reasonably expected to assist the interaction between **1** and the negatively charged peptide-a, whereas the repulsive interaction greatly lessens the affinity to the positively charged peptide-b. In the case of the almost neutral peptide-c, such an additional interaction does not operate. The order of the affinity constants for these peptides in our experiments is consistent with this explanation. These results demonstrate that the

Table 1. Summary of the Apparent Association Constant (M^{-1}) of Various Receptors to Peptides Determined by the Peptide-Induced Fluorescence Change

receptor/peptide	1	2	3
a	1.8×10^7	8.7×10^6	3.9×10^4
b	—[a]	—[a]	—[b]
c	1.2×10^4	2.1×10^5	—[b]
d	—[a]	—[a]	—[b]

[a] Since the fluorescence change did not take place, we cannot evaluate the association constant. [b] Not determined.

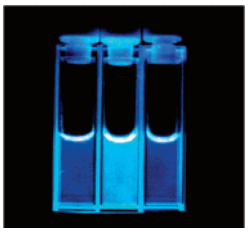


Figure 4. Photograph of the increased emission of the receptor **1** in the presence of phosphorylated peptide-a (middle); the solution of **1** only (left); **1** in the presence of nonposphorylated peptide-d (right).

present receptor can selectively discriminate the peptide sequence, as well as the phosophorylation/dephosphorylation event. Almost the same capability is obtained for the receptor **2**. On the other hand, the low affinity of the mononuclear receptor **3** was evaluated even for the Glu-rich peptide-a, suggesting that the cooperative action of two Zn(Dpa) sites of **1** and **2** is effective for the strong binding (See Chart 2). A typical photograph of the fluorescence change of the receptor **1** upon addition of peptide-a is displayed in Figure 4. Apparently, the blue fluorescence is greatly intensified by the phosphorylated peptide-a, whereas the nonphosphorylated peptide-d does not induce the fluorescence change. It is demonstrated that one can simply distinguish such selectivity by the naked eye.

To our knowledge, these are the first chemosensors that can selectively bind and sense a phosphorylated peptide surface of biological importance under aqueous conditions.[12] We believe that the elaborated modification of this scaffold facilitates specificity in the recognition and sensing of a variety of phosphorylated protein surface. Furthermore, since zinc cation is physiologically abundant,[13] the present strategy based on coordination chemistry is anticipated to be the first step toward rational design of the molecular modulators which can regulate the cell signaling pathway through blocking a protein−protein surface interaction.

Acknowledgment. We are grateful to Professor S. Shinkai and Dr. S. Tsukiji for their fruitful discussion in the initial stage of this research.

Supporting Information Available: Experimental details for synthesis of the receptors and the phosphorylated peptides, and for the measurements for ^{1}H NMR and fluorescent titrations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) *Chem. Rev.* **2001**, *101*, issue 8: *Protein Phosphorylation and Signaling*. (b) Sefton, B. M.; Hunter, T. *Protein Phosphorylation*; Academic Press: New York, 1998.

(2) (a) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515. (b) Mason, W. T. *Fluorescent and Luminescent Probes for Biological Activity*; Academic Press: London, 1999. (c) Kimura, E.; Koike, T. *Chem. Soc. Rev.* **1998**, *27*, 189. (d) Burdette, S. C.; Walkup, G. K.; Spingler, B.; Tsien, R. Y.; Lippard, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 7831. (e) Hirano, T.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2000**, *122*, 12399. (f) Kraynov, V. S.; Chamberlain, C.; Bokoch, G. M.; Schwartz, M. A.; Slabaugh, S.; Hahn, K. M. *Science* **2000**, *290*, 333. (g) Hamachi, I.; Nagase, T.; Shinkai, S. *J. Am. Chem. Soc.* **2000**, *122*, 12065. (h) Nagase, T.; Shinkai, S.; Hamachi, I. *Chem. Commun.* **2001**, 229.

(3) (a) Beer, P. D.; Gale, P. A. *Angew. Chem., Int. Ed.* **2001**, *40*, 486. (b) Beer, P. D.; Cadman, J. *New. J. Chem.* **1999**, *23*, 347. (c) Schneider, S. E.; O'Neil, S. N.; Anslyn, E. V. *J. Am. Chem. Soc.* **2000**, *122*, 542. (d) Deetz, M. J.; Smith B. D. *Tetrahedron Lett.* **1998**, *39*, 6841. (e) Hosseini, M. W.; Blacker, A. J.; Lehn, J.-M. *J. Am. Chem. Soc.* **1990**, *112*, 3896.

(4) (a) Vance, D. H.; Czarnik, A. W. *J. Am. Chem. Soc.* **1994**, *116*, 9397. (b) Huston, M. E.; Akkaya, E. U.; Czarnik, A. W. *J. Am. Chem. Soc.* **1989**, *111*, 8735.

(5) (a) Wilcox, D. E. *Chem. Rev.* **1996**, *96*, 2435. (b) Lipscomb, W. N.; Sträter, N. *Chem. Rev.* **1996**, *96*, 2375.

(6) Recent advancement in model system of multinuclear phosphatase: (a) Kimura, E. *Curr. Opin. Chem. Biol.* **2000**, *4*, 207. (b) Molenveld, P.; Engbersen F. J.; Reinhoudt, D. N. *Chem. Soc. Rev.* **2000**, *29*, 75. (c) Williams, N. H.; Takasaki, B.; Wall, M.; Chin, J. *Acc. Chem. Res.* **1999**, *32*, 485. (d) Kaminskaia, N. V.; He, C.; Lippard, S. *J. Inorg. Chem.* **2000**, *39*, 3365.

(7) These compounds were identified by mass spectrometry and elemental analysis. See Supporting Information.

(8) The decrement of the cationic character of the pyridine rings of Dpa is induced by the binding of the phosphate anion to the zinc center so as to suppress the PET quenching of the photoexcited anthracene by the cationic pyridines. This may be another plausible mechanism for the fluorescence intensification of **1** and **2** upon binding to the phosphorylated species. De Silva, A. P.; Gunarante, H. Q. N.; Lynch, P. M. L. *J. Chem. Soc., Perkin Trans. 2* **1995**, 685.

(9) On the basis of CD spectra, these peptides were found to be random coils in the absence and presence of the receptors.

(10) Pinna, L. A.; Ruzzene, M. *Biochim. Biophys. Acta* **1996**, *1314*, 191.

(11) (a) Songyang, Z.; Cantley, L. C. *Trends Biochem. Sci.* **1995**, 470. (b) Songyang, Z.; Carraway, K. L., III; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammadi, M.; Schlessinger, J.; Hubbard, S. R.; Smith, D. P.; Eng, C.; Lorenzo, M. J.; Ponder, B. A. J.; Mayer, B. J.; Cantley, L. C. *Nature* **1995**, *373*, 536.

(12) Protein surface recognition in aqueous medium: (a) Mito-oka, Y.; Tsukiji, S.; Hiraoka, T.; Kasagi, N.; Shinkai, S.; Hamachi, I. *Tetrahedon Lett.* **2001**, *42*, 7059. (b) Hamachi, I.; Kasagi, N.; Nagase, T.; Kiyonaka, S.; Mito-oka, Y.; Shinkai, S. *Chem. Lett.* **2001**, 16. (c) Takashima, H.; Shinkai, S.; Hamachi, I. *Chem. Commun.* **1999**, 2345. (d) Hamachi, I.; Fujita, A.; Kunitake, T. *J. Am Chem. Soc.* **1997**, *119*, 9096. (e) Peczuh, M. W.; Hamilton, A. D. *Chem. Rev.* **2000**, *100*, 2479. (f) Orner, B. P.; Ernst, J. T.; Hamilton A. D. *J. Am. Chem. Soc.* **2001**, *123*, 5382. (g) Blaskovich, M. A.; Lin, Q.; Delarue, F. L.; Sun, J.; Park, H. S.; Coppola, D.; Hamilton, A. D.; Sebti, A. M. *Nature Biotechnol.* **2000**, *18*, 1065. (h) Jain, R. K.; Hamilton, A. D. *Organic Lett.* **2000**, *2*, 1721. (i) Fazal, M. A.; Roy, B. C.; Sun, S.; Mallik, S.; Rodgers, K. R. *J. Am. Chem. Soc.* **2000**, *123*, 6283. Peptide surface recognition in water−organic solvent mixture: (j) Orner, B. P.; Salvatella, X.; Quesada, J. S.; de Mendoza, J.; Giralt, E.; Hamiltom, A. D. *Angew. Chem., Int. Ed.* **2002**, *41*, 117. (k) Haack, T.; Peczuh, M. W.; Salvatella, X.; Sánchez-Quesada, J.; de Mendoza, J.; Hamilton, A. D.; Giralt, E. *J. Am Chem. Soc.* **1999**, *121*, 11813. (l) Tabet, M.; Labroo, V.; Sheppard, P.; Sasaki, T. *J. Am. Chem. Soc.* **1993**, *115*, 3866.

(13) Valle, B. L.; Falchuk, K. H. *Physiol. Rev.* **1993**, *73*, 79.

JA025761B