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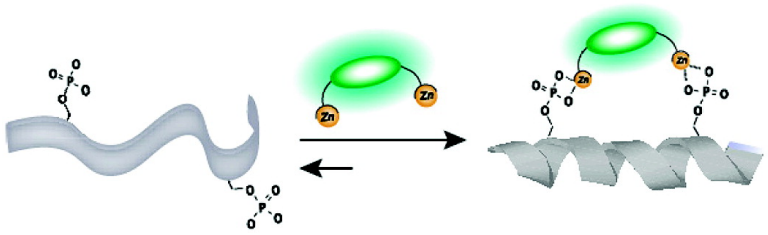
J. Am. Chem. Soc., **2003**, 125 (34), 10184-10185• DOI: 10.1021/ja036317r • Publication Date (Web): 05 August 2003



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Published on Web 08/05/2003

Cross-Linking Strategy for Molecular Recognition and Fluorescent Sensing of a Multi-phosphorylated Peptide in Aqueous Solution

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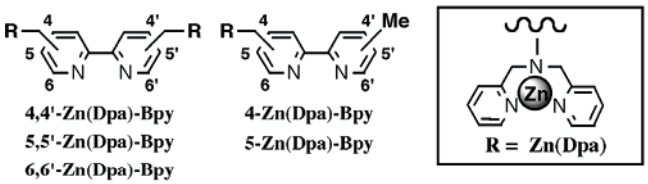
In the research field of molecular recognition, selective binding of large biomolecules such as peptides/proteins, polysaccharides, or oligonucleic acids in aqueous solution is becoming an important goal, because of its potential impact in the diagnostic and pharmaceutical applications. Compared to the great progress in DNA recognition pioneered by Dervan and co-workers,[1] protein surface recognition is not yet well developed.[2] A great advance in molecular cell biology and biochemistry unveiled the fact that kinase-catalyzed phosphorylation of Ser, Thr, or Tyr located on a protein surface is an essential event for switching enzymatic activities and/or regulation of protein−protein interactions in signal transduction cascades of living cell.[3] Therefore, the selective recognition and sensing of a phosphorylated protein surface is strongly required not only for elucidation of protein−protein recognition at the molecular level, but also for regulation of signal transduction through protein surfaces. We recently developed an artificial fluorescent chemosensor for mono-phosphorylated peptides on the basis of coordination chemistry.[4] Here we describe a new strategy for molecular recognition of a multi-phosphorylated peptide using intrapeptide cross-linking.[5] It is expected that multi-point interactions can enhance the binding selectivity for a multi-phosphorylated peptide with a specific distance.

In the receptor design, dipicolylamine zinc(II) complex (Zn(Dpa)) is recruited as a binding module for phosphorylated amino acids, according to our previous report.[4] Two Zn(Dpa) modules are connected with 2,2′-bipyridine as a spacer in 4,4′-, 5,5′-, or 6,6′-substitution, by which the distance between two Zn centers can be adjusted to a target peptide having two phosphorylated amino acids (Chart 1).[6]

The validity of the cross-linking strategy was initially tested using three phosphorylated α-helical model peptides (pS-12,16, pS-9,16, and pS-5,16) containing two pS (phosphorylated serine) residues with varied distances, and a mono-phosphorylated peptide (pS-16) (Chart 1).[7] As shown in Figure 1a, the helix content greatly increases upon addition of an appropriate artificial receptor, which is monitored by CD spectroscopy. Rough screening for the capability of a series of the receptors to bind to these peptides was conducted using the difference CD value (Figure 1b). The dinuclear receptors, with the exception of the 6,6′-substituent,[8] considerably induced the α-helix content of peptides having p-Ser, whereas these did not affect the secondary structure of the mono-phosphorylated control peptide. A mononuclear receptor, 5-Zn(Dpa)-Bpy or zinc cation itself, on the other hand, less effectively induced the helical conformation. These results suggest that a two-point interaction between two Zn(Dpa) modules of the receptor and two p-Ser residues of the corresponding peptide causes an increase of α-helix

Chart 1



pS-5,16 : Ac-**AEAApSKEAAAKEAAApSA**-NH_2
pS-9,16 : Ac-**AEAAAKEApSAKEAAApSA**-NH_2
pS-12,16 : Ac-**AEAAAKEAAAKpSAAApSA**-NH_2
pS-16 : Ac-**AEAAAKEAAAK EAAApSA**-NH_2

IRK-2P : Ac-**TRDIpYETDpYYRK**-NH_2
IRK-1P : Ac-**TRDIpYETDYYRK**-NH_2
IRK-0P : Ac-**TRDIYETDYYRK**-NH_2

conformation via cross-linking stabilization. The most significant CD increase was observed in the combination of 5,5′-Zn(Dpa)-Bpy with pS-9,16-pep, in which 30% helical conformation was induced from an almost random conformation. Since the 1:1 stoichiometry was confirmed by the Job plot, one can evaluate the binding constant ($(1.6 \pm 0.20) \times 10^6\ M^{-1}$) by detailed CD titration (see Supporting Information). The ^{31}P NMR experiment for the receptor 5,5′-Zn(Dpa)-Bpy and pS-9,16 peptide indicates that two distinct peaks (1.48 and 1.25 ppm) due to 9-pS and 16-pS shifted and merged into a single broad peak at 1.06 ppm upon addition of the receptor. This is evidence that the phosphate sites in the peptide are directly involved in the receptor binding.

Since the present 5,5′-Zn(Dpa)-Bpy is fluorescent,[9] we subsequently examined the fluorescence spectral change of the receptors upon addition of a series of peptides. Figure 2a shows a typical fluorescence change of 5,5′-Zn(Dpa)-Bpy by pS-9,16. The emission

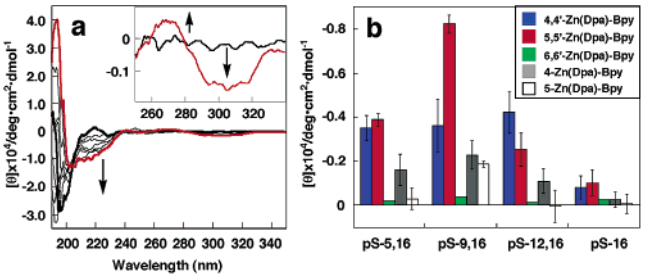


Figure 1. (a) CD spectral change of pS-9,16 (20 μM) upon addition of 5,5′-Zn(Dpa)-Bpy (0−3 equiv). (Inset) The induced CD due to the bipyridyl moiety. (b) θ value change (222 nm) upon addition of 2 equiv of the Zn-(Dpa)-based receptors to the corresponding peptides (20 μM). Conditions: 10 mM borate buffer (pH 8.0) at 10 °C.

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10.1021/ja036317r CCC: $25.00

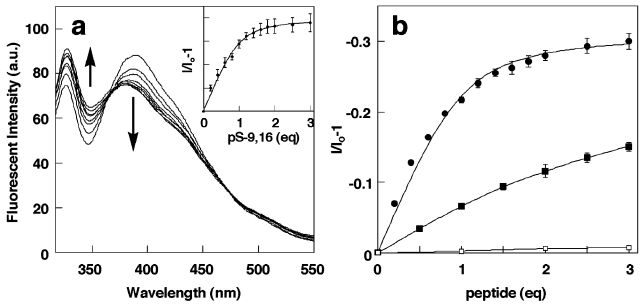

Figure 2. (a) Fluorescence spectral change of 5,5′-Zn(Dpa)-Bpy (5 μM) upon the addition of pS-9,16 (0−3 equiv) Conditions: 10 mM borate buffer (pH 8.0) at 20 °C. (Inset) Fluorescent titration curve (λ_{em} = 389 nm) of Zn(Dpa)-5,5′-Bpy (5 μM) with pS-9,16. (b) Fluorescent titration curve of 5,5′-Zn(Dpa)-Bpy (λ_{em} = 389 nm) with a series of IRK peptides: IRK-2P (●), IRK-1P (■), IRK-0P (□). Conditions: 10 mM borate buffer, 50 mM NaCl (pH 8.0) at 20 °C.

Table 1. Binding Constats of 5,5′-Zn(Dpa)-Bpy with Various Peptides Determined from the Peptide-Induced Fluorescence Change

peptide	$K^a/\times 10^6$ M^{-1}	peptide	$K^a/\times 10^6$ M^{-1}
pS-5,16	0.75 ± 0.09	IRK-2P	1.7 ± 0.20
pS-9,16	2.0 ± 0.46	IRK-1P	0.07 ± 0.001
pS-12,16	0.72 ± 0.10	IRK-0P	*b*
pS-16	0.05 ± 0.02		

[a] The errors were calculated using the data of three titration experiments. [b] No fluorescence change was observed.

intensity at 389 nm gradually decreases and is saturated by addition of pS-9,16-peptide, which gives the binding constant of (2.0 ± 0.46) × 10^6 M^{-1}. This value agrees well with the value obtained by the above-mentioned CD titration. Similar fluorescence changes were observed for other peptides, and the binding constants are summarized in Table 1. It is clear that the binding affinity of 5,5′-Zn-(Dpa)-Bpy is greatly dependent on the number of pS; that is, the selectivity of the di-phosphorylated peptide over that of the mono-phosphorylated one is more than 10-fold. On the other hand, the binding selectivity among di-phosphorylated peptides seems moderate. Although the tightest binding was observed for pS-9,16, consistent with the CD screening result, the difference is only 3-fold in magnitude compared to the binding for pS-12,16 and pS-5,16. This is probably ascribed to both the fluctuation of the peptide conformation and the freedom of motion of the methylene unit of the receptor. A molecular modeling study of the receptor suggested that the distance between two zinc centers is 11−13 Å, which is roughly comparable to the distance between p-Ser (*i*) and p-Ser (*i*+7).

Next, we evaluated the capability of the chemosensors to detect naturally occurring peptides containing two phospho-amino acids separated by more than 10 Å. Insulin receptor kinase (IRK) is reported to be given a hormone-induced hyper-phosphorylation which activates IRK, and as a result, the subsequent serial signal transduction cascade is switched on.[10] In the activation loop of IRK, di-, mono-, and non-phosphorylated peptide segments (1154−1165; IRK-2P, IRK-1P, IRK-0P, respectively) were prepared as targets for 5,5′-Zn(Dpa)-Bpy.[11] Similar to Figure 2a, the fluorescence of the receptor 5,5′-Zn(Dpa)-Bpy at 389 nm lessened upon IRK-2P addition (see Supporting Information). Such a fluorescence change is completely similar to that for the model peptide pS-9,16, and the fluorescence Job plot demonstrated a 1:1 ratio (see Supporting Information), strongly suggesting that 5,5′-Zn(Dpa)-Bpy is bound to IRK-2P in a cross-linking manner. The titration curve (Figure 2b) gave a binding constant of (1.7 ± 0.20) × 10^6 M^{-1}. This value is more than 20-fold greater than that for IRK-1P (see Table 1). A negligible change occurred fluorometrically in the case of IRK-0P. The order of the binding constant is again in good agreement with the model peptide. The exciton coupling of the induced CD of the bipyridyl moiety clearly appeared in the 1:1 complex of dinuclear 5,5′-Zn(Dpa)-Bpy with IRK-2P, whereas the CD peak was not observed with the mono-nuclear 5-Zn(Dpa)-Bpy (see Supporting Information). The positive Cotton effect may suggest that the two pyridine rings of 5,5′-Zn(Dpa)-Bpy chirally tilted in the presence of peptide.[12]

In conclusion, we developed a new fluorescence chemosensor selective for doubly phosphorylated peptides that can be used in neutral aqueous solution using intrapeptide cross-linking. This strategy may be applied to other heterolytically modified peptides such as His(Glu, Lys)/pS(Y) peptides or glycosylated Asn(or S)/pS peptides, and thus expand the application field of artificial receptors. We are now studying ways to apply this type of receptors to kinase activity assay or regulation of protein−protein interactions.

Supporting Information Available: Experimental details for synthesis of the Zn complexes and the phosphorylated peptides, and for the measurement of CD, fluorescence, and ^{31}P NMR (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA036317R