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Anion Sensor-Based Ratiometric Peptide Probe for Protein Kinase Activity

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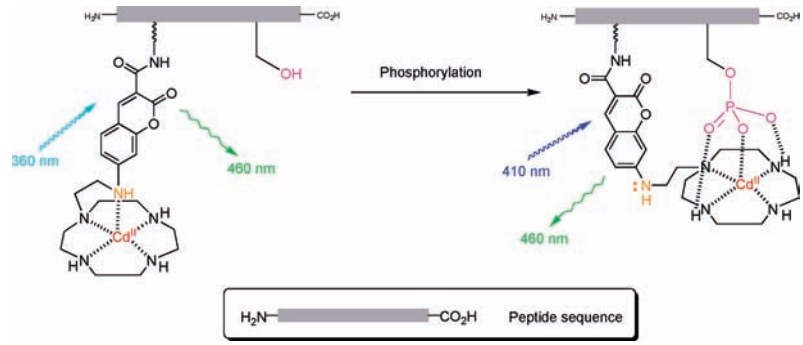
ABSTRACT



A new fluorescent sensor consisting of Cd^{II}-cylcen appended aminocoumarin and a substrate peptide for protein kinase A (PKA) has been designed. Upon phosphorylation by PKA, the metal complex moiety binds to a phosphorylated residue, which in turn displaces the coumarin fluorophore, and this event results in ratiometric change of excitation spectrum in neutral aqueous solution.

Signal transduction pathways provide mechanisms for transducing external signals to intracellular biological responses. Protein kinases modulate the activity of their target proteins by phosphorylating serine, threonine and tyrosine residues within the intact proteins in these pathways. A great number of kinases have been discovered, and the characterization of their roles in complicated signaling pathways is now a very active research area.[1] The development of an analytical tool that can enable monitoring of the temporal and spatial dynamics of cellular kinases would therefore contribute substantially to a better understanding of signal transduction mechanisms.[2]

Various approaches to monitor the activities of protein kinases have been made,[3,4] one of which is the use of fluorophore-labeled peptide substrates.[5] Traditional peptide probes contain a polarity-sensitive fluorophore near the site

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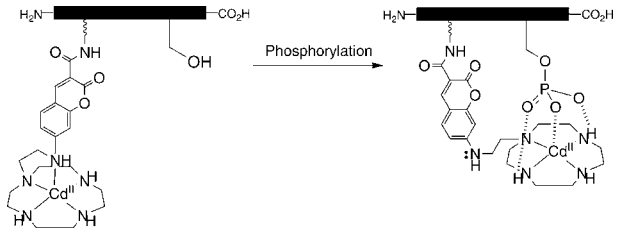
of phosphorylation, and this serves to signal the change of environment upon phosphorylation. The groups of Lawrence and Imperiali have developed chelator-appended fluorescent peptides for monitoring kinase activities.[6] Upon phosphorylation, these peptides show a significant fluorescence intensity increase owing to the formation of divalent alkaline earth metal complexes coordinated to the newly generated phosphate group and the fluorophore.

Fluorescence measurement at a single wavelength without much shift of either the excitation or emission wavelength can be influenced by artifacts associated with the microscopic imaging system. To reduce the influence of such factors, ratiometric measurement is utilized, namely, simultaneous recording of the fluorescence intensities at two wavelengths and calculation of their ratio.[7] For this approach, probes that signal phosphorylation via a shift of either excitation or emission wavelength are required.

We have designed a fluorescent anion sensor, consisting of 7-aminotrifluoromethylcoumarin as a fluorescent reporter and Cd^{II}-cyclen (1,4,7,10-tetraazacyclododecane) as an anion host.[8] This sensor molecule can detect phosphate anion species, such as pyrophosphate, with high sensitivity in aqueous neutral solution. As an extension of the anion sensor concept, we have newly designed an anion sensor-appended peptide substrate for protein kinases. Here we describe the sensing of a kinase-mediated phosphorylation event by a fluorescent peptide sensor. This novel class of peptide probe exhibited a shift of excitation spectrum upon phosphorylation, enabling ratiometric measurement of kinase activity. This technique can provide more precise data than measurement at a single wavelength, canceling out the influence of variations in instrument efficiency, content of effective dye, and so forth.

The operational concept of the peptide sensor is schematically presented in Scheme 1. This peptide sensor consists of

Scheme 1. Schematic Representation of our Peptide Sensor for Phosphorylation

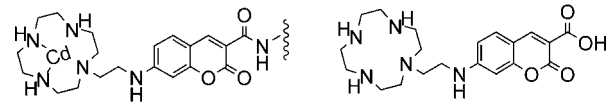


an anion sensor and a phosphorylation target peptide sequence. The sensing moiety is positioned near the target hydroxyl amino acid residue. In neutral aqueous solution, Cd^{II} of the cyclen complex is coordinated by the four nitrogen atoms of cyclen and the aromatic 7-amino group of coumarin.[9] When a negatively charged phosphate group coordinates to Cd^{II} as the fifth ligand, the aromatic 7-amino group is displaced from the metal. The anion sensor signals this replacement, because the increase of electron density of the 7-amino group induces a red shift of the excitation spectrum. We have designed peptide sensor **1** for protein kinase A (PKA) as shown in Scheme 2. The sequence of the peptide

Scheme 2. Sequence of Peptide Sensor and its Phosphorylated Standard Employed in This Study

Sensor-$(CH_2)_5$-LRRA<u>S</u>LG-$CONH_2$ **1**

Sensor-$(CH_2)_5$-LRRAp<u>S</u>LG-$CONH_2$ **1P**



Sensor **2**

sensor is known as Kemptide and has been shown to be a good substrate for the kinase.[10] The sensing moiety is positioned at the N-terminus of the peptide through an alkyl tether, enabling recognition of a phosphorylated serine residue. We also designed a phosphorylated sensor **1P** to estimate preliminarily the extent of spectral change upon phosphorylation.

The cyclen-appended 7-aminocoumarincarboxylic acid **2** was synthesized according to the established procedure.[8] The peptide sequence was synthesized using Fmoc solid-phase chemistry on an automated peptide synthesizer and the ligand **2** was manually coupled to the amino linker. The resulting peptide conjugate was metalated with $Cd(ClO_4)_2$ to give the desired peptide sensor **1**. Phosphorylated peptide sensor **1P** was prepared by protein kinase-mediated phosphorylation of the peptide conjugate followed by metalation with Cd^{II}. The structures of **1** and **1P** were confirmed by MALDI-TOF MS (matrix assisted laser desorption/ionization-time-of-flight mass spectrometry) and quantitative amino acid analysis.

We tested the sensing ability of peptide sensor **1** by comparing the excitation spectrum with that of the phosphorylated product, **1P** (see Supporting Information). Upon phosphorylation, the excitation intensity at 360 nm decreased, whereas the intensity at 410 nm increased. The ratio of the excitation intensities (410 nm/360 nm) changed

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1.8-fold (from a value of 0.54 to 0.96), demonstrating that peptide phosphorylation can be detected with an anion sensor.[11]

To investigate the utility of the compound as a fluorescent probe for protein kinases, we measured the time-dependent change of the excitation spectrum of **1** treated with ATP (adenosine 5′-triphosphate) and PKA catalytic subunit (Figure 1). The phosphorylation reaction

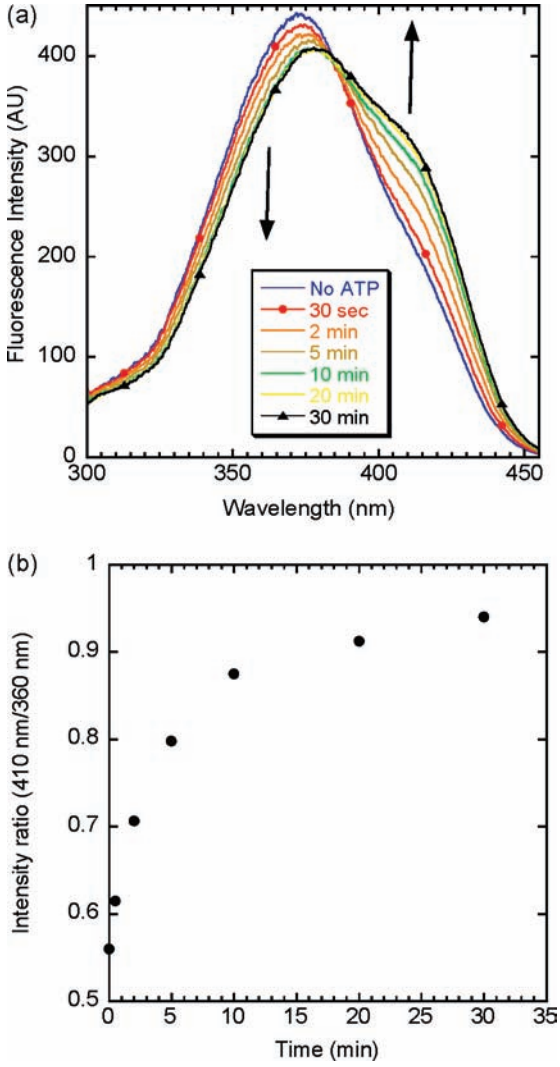

Figure 1. (a) Time course of the excitation spectra of **1** treated with PKA catalytic subunit. The peptide sensor **1** (1.3 μM) was incubated in 50 mM HEPES (pH 7.4), 5 mM $Mg(OAc)_2$ containing 4.3 nM catalytic subunit and 3.3 μM ATP at 23 ± 0.1 °C. (b) Plot of intensity ratio (410 nm/360 nm) versus reaction time.

was initiated by the addition of ATP to a mixture of **1** and the catalytic subunit. Though ATP strongly coordinates to the Cd^{II} complex of the anion sensor, addition of this organic polyanion had no significant effect on the excitation spectrum under the conditions employed.[12] As can be seen from Figure 1, the excitation spectra of **1** changed ratiometrically; the intensity at 360 nm decreased with a concomitant intensity increase at 410 nm. The ratio of excitation intensity (410 nm/360 nm) increased 1.7 fold after 30 min reaction time, and this is similar to the value obtained by comparison of the excitation spectra of authentic **1** and **1P**. The phosphorylation reaction was accelerated by increasing the quantity of kinase employed for the reaction (see Supporting Information).

We further carried out an inhibition experiment using the heat-stable inhibitor protein of PKA (PKI), which acts competitively with respect to the phosphoryl-accepting substrate (Figure 2).[13] Dose-dependent inhibition of kinase

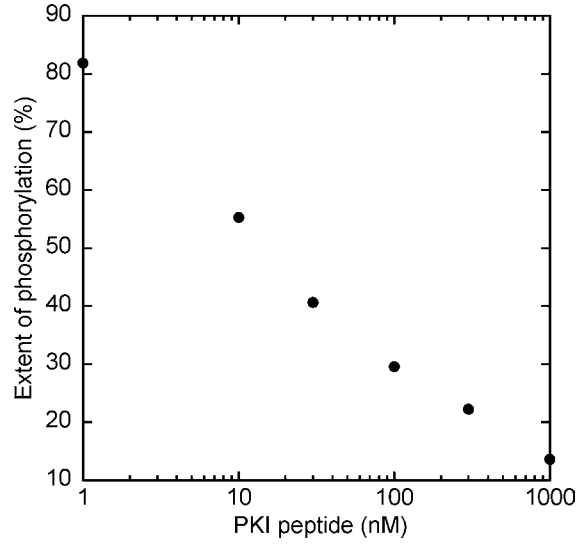

Figure 2. Titration of PKA-mediated phosphorylation of **1** with PKI peptide. The phosphorylation reaction was carried out in 50 mM HEPES (pH 7.4), 5 mM $Mg(OAc)_2$ containing 1.0 μM **1**, 4.3 nM catalytic subunit, 3.3 μM ATP and various amounts of PKI peptide at 23 ± 0.1 °C. Extent of phosphorylation (%) was determined by comparing the intensity ratio increase at the early phase of the reaction (0−5 min) with that of the control.

activity by PKI peptide was observed for phosphorylation of **1** with an IC_{50} (half maximal inhibitory concentration) of ca. 15 nM under the conditions employed. This result indicates that the ratiometric spectral change is caused by PKA-mediated phosphorylation of **1**.[14]

In conclusion, we have developed a new fluorescent probe for protein kinase based on the anion sensing principle. It has been demonstrated that this peptide probe can be used

(11) A significant shift of the absorption peak was also observed for the Cd^{II} complex of methylated compound **2** upon addition of pyrophosphate anion. Titration of the complex with pyrophosphate gave the Kd value of 53 μM.

(12) The addition of more than three equivalents of ATP to the peptide sensor **1** induced an excitation change at two wavelengtth (360 and 410 nm), which indicates the coordination of ATP to the metal complex moiety.

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to continuously monitor kinase-mediated phosphorylation through intensity measurements at two wavelengths. This peptide sensor might serve as the basis for a range of anion sensor-based phosphorylation probes for many different protein kinases.

(14) Actual phosphorylation of peptide sensor **1** was confirmed by analyzing the reaction mixture, using C_{18} reverse-phase HPLC (high-performance liquid chromatography). Time-dependent production of phosphorylated product was observed when the sensor **1** was exposed to PKA catalytic subunit. The phosphorylated product co-eluted with authentic standard **1P** from the HPLC column.

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Supporting Information Available: Synthesis of anion sensor and peptide conjugate, fluorescence experiment, protein kinase assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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