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Fluorogenic assay of alkaline phosphatase activity based on the modulation of excited-state intramolecular proton transfer

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ABSTRACT

A new fluorogenic substrate **1**, which enables the fast and quantitative analysis of alkaline phosphatase activity, has been developed. Selective enzymatic hydrolysis of **1** instantly generated fluorescent compound **2** in aqueous media, which undergo an excited-state intramolecular proton transfer process, resulting in a remarkable fluorescence turn-on signal with an unusually large Stokes shift.

Introduction

Alkaline phosphatases (ALPs), which catalyze hydrolysis or transphosphorylation of a wide variety of phosphate compounds, are a group of enzymes found in a variety of tissues (intestine, liver, bone, kidney, and placenta) of nearly all living organisms.[1–3] This enzyme has been extensively used as a biomarker in enzyme immunoassays and molecular biology.[4] ALP is also one of the most commonly assayed enzymes in routine clinical practice. Because of its abnormal level of serum, ALP is an important diagnostic indicator of many human diseases, such as bone diseases (osteoblastic bone cancer, Paget's disease, and osteomalacia) and liver diseases (cancer, hepatitis, and obstructive jaundice).[5–7] Therefore, a sensitive and continuous assessment of ALP activity would be extremely valuable for providing effective diagnostic approaches and/or therapeutic targets in biomedical research as well as for offering the possibility of studying the role of this enzyme in various physiological and biological processes.

Due to unparalleled sensitivity, simplicity, rapid implementation, and applicability to high throughput screening, fluorescence-based probes are promising tools to detect the catalytic activity of ALP. The most common fluorescent probes for monitoring ALP activity are coumarin- and fluorescein-based fluorogenic substrates, in which an increase in fluorescence intensity occurs upon the enzyme-catalyzed hydrolysis of a phosphate group.[8–10] These probes offer high sensitivity and can be used in a continuous assay of ALP activity; however, their water-soluble fluorescent products often excret out of cells, limiting their use in detecting endogenous ALP activities. In case of fluorescein-based substrates, enzymatic products exhibit a fluorescence emission with small Stokes shifts, resulting in high background signals and interferences (self-absorption, etc.).

Intramolecularly hydrogen-bonded molecules such as *o*-hydroxybenzoxazole, -benzimidazole, and -benzothiazole often undergo an excited-state intramolecular proton transfer (ESIPT), in which rapid photoinduced proton transfer results in the formation of two tautomeric forms.[11,12] Such organic fluorescent compounds harnessing an ESIPT process have attracted great attention due to their distinct photophysical properties, which facilitate a wide variety of sensory applications for ions, chemicals, and biomolecules.[13–15]

2-(2′-Hydroxyphenyl)-4-phenylthiazole **2** is known to exhibit an ESIPT process.[16] In the ground state, compound **2** exists in the enol form (**2**-E). Irradiation of chromophore **2** converts the enol (**2**-E*) to the excited-state keto species (**2**-K*) in the subpicosecond time scale (Scheme 1a). The resulting emission from **2**-K* exhibits an unusually large Stokes shift. Since **2**-K* emission occurs in a region of the spectra that is clear of interferences (self-absorption, etc.), we sought to explore the turn-on fluorescence probes that rely on an ESIPT process. In this paper, we report an ESIPT-controlled fluorescence turn-on probe for monitoring ALP activity in aqueous media.

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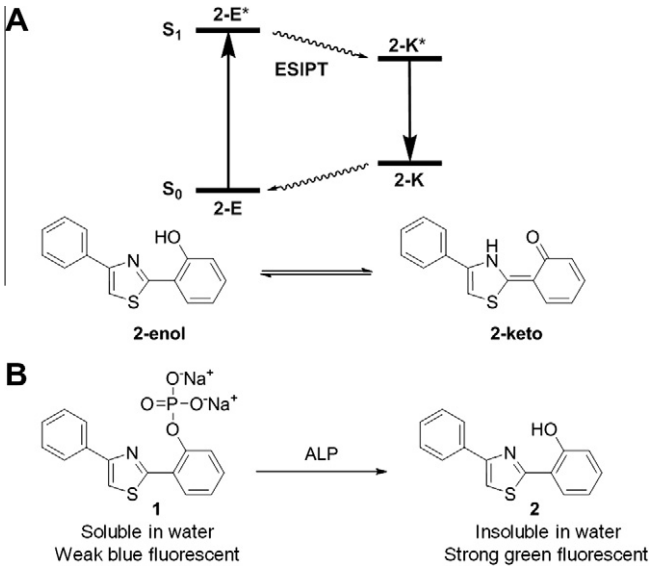

Scheme 1. (a) General mechanism of the ESIPT process and chemical structures of the **2-enol** and **2-keto** forms in the ESIPT process of compound **2**. (b) Illustration of the fluorometric assay for ALP using ESIPT-based probe **1**.

Scheme 1b depicts our design rationale for ALP assays based on an ESIPT fluorophore (**2**). Fluorogenic substrate **1** was expected to display enol emission (E*) exclusively due to the inhibition of the ESIPT process. In addition, the excited probe **1** was expected to have less efficient emission because of the fast non-radiative decay of the singlet excited state facilitated via internal bond rotations. However, ALP-catalyzed cleavage of the phosphate group in probe **1** induces a fast generation of ESIPT-product **2**, and increases the quantum yields of the corresponding keto-tautomer emission upon photoexcitation.

Probe **1**, as a new substrate for ALP, was synthesized in a moderate yield according to Scheme S1. 2-(2′-Hydroxyphenyl)-4-phenylthiazole **2** was phosphorylated with diethyl chlorophosphate and NaH in THF to afford phosphate triester **3**. Selective hydrolysis of **3** was acheived with bromotrimethylsilane to give the water-soluble probe **1** after neutralization with NaOH.

The UV–visible absorption spectrum of probe **1** in water displayed a strong electronic transition at $\lambda_{max,abs}$ = 315 nm (ε = 17380 M^{-1} cm^{-1}), and the emission spectrum showed a peak maximum at 385 nm, which is characteristic of an enol-like emission (Fig. 1). To validate the proposed sensing mechanism, the photophysical properties of 2-(2′-hydroxyphenyl)-4-phenylthiazole **2**—the expected product after reaction with ALP—were examined in water containing 2% EtOH as a cosolvent. The UV–visible absorption spectrum of **2** displayed a maximum at 340 nm (ε = 13750 M^{-1} cm^{-1}) and the emission spectrum of **2** showed a strong keto tautomer emission (~522 nm) resulting from the ESIPT. The emission quantum yields (Φ_F) in the aqueous solution were 0.03 for probe **1** and 0.12 for compound **2**, relative to that of quinine sulfate in 0.1 M H_2SO_4. Enhanced emission efficiency and a large bathochromic shift in the emission maxima of **2** with respect to those observed in probe **1** indicated that a higher signal-to-noise ratio can be obtained from the conversion of probe **1** to **2** upon reaction with ALP.

Upon incubation of probe **1** (20 μM) with ALP (50 nM) in a buffered aqueous solution (10 mM Tris–HCl, pH 9.0, 37 °C), the absorption spectrum showed a clear bathochromic shift from 316 nm to 339 nm, indicating the formation of compound **2**. The characteristic ESIPT-fluorescence signal of **2** at 521 nm immediately arose and increased significantly with time (Fig. 2) before reaching a plateau within 10 min. A remarkable increase (ca. 455-fold) in fluorescence intensity at 521 nm was obtained upon incubation with ALP (50 nM) for 10 min. The weak blue emissive nature of probe **1** and the bright green fluorescence upon cleavage of phosphate group of **1** were clearly visualized, as shown in Figure 2.[17]

We further investigated the ALP-catalyzed hydrolysis of probe **1** as a function of incubation time using different enzyme concentrations. Figure 3a illustrates the increase in fluorescence intensity at 525 nm for solutions with different ALP concentrations (0–50 nM), in which fluorescence intensity was measured every 1 min. As expected, the fluorescence intensity increased with increasing [ALP] within this concentration range. The increase in fluorescence intensity at 525 nm was shown to be quantitatively related to the increase of ESIPT-product **2**, thus allowing real-time monitoring of enzyme activity (Fig. 3B). We then found that ALP could be assayed with a sensitivity limit as low as 0.25 nM of ALP.

We then conducted kinetic measurements for the enzymatic hydrolysis reaction of fluorogenic substrate **1** and determined kinetic parameters using Lineweaver–Burk analysis, yielding values

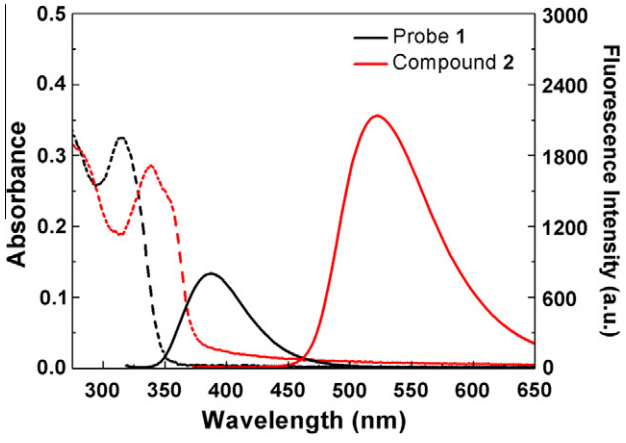

Figure 1. Absorption (dashed lines) and emission spectra (solid lines) of probe **1** (black) and product **2** (red) in an aqueous solution at 25 °C. Probe **1** in water and **2** in 2% EtOH/H_2O (v/v). Excited at 300 nm for probe **1** and at 340 nm for product **2**. [**1**] = [**2**] = 20 μM.

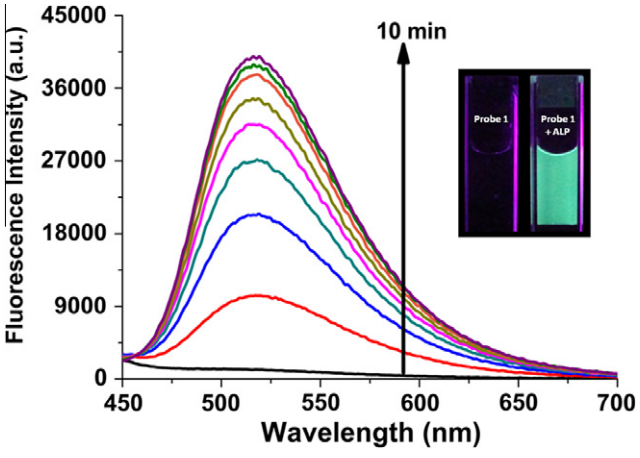

Figure 2. Emission spectra of probe **1** (20 μM) upon treatment with ALP (50 nM) for different time periods (0–10 min) in Tris–HCl buffer (10 mM, pH 9.0, 37 °C). The spectra were obtained every 1 min. Excited at 340 nm. Inset: Photographs of probe **1** (20 μM) in the absence (left) and presence (right) of ALP (50 nM) after incubation for 10 min at 37 °C under UV light (365 nm) illumination.

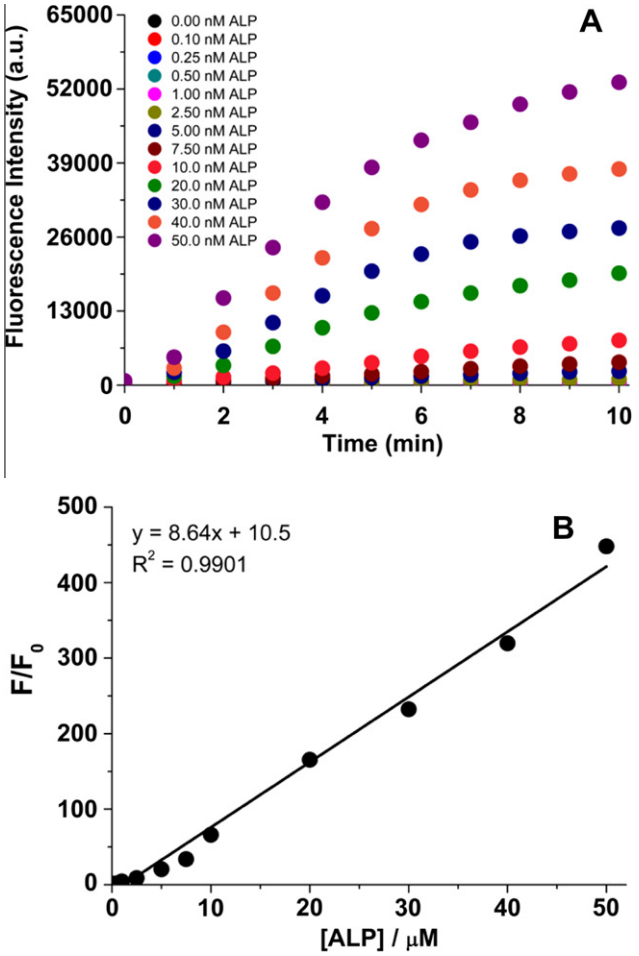

Figure 3. (A) Increase in fluorescence intensity at 525 nm, recorded every 1 min (0–10 min) during real-time turn-on assay of probe **1** (20 μM) under varying concentrations (0–50 nM) of ALP in Tris–HCl buffer (10 mM, pH 9.0) at 37 °C. Excited at 340 nm. (B) Relative fluorescence intensity at 525 nm as a function of concentration of ALP. Incubation time = 10 min. F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and the presence of ALP, respectively.

of the Michaelis constant K_m = 1.90 μM and the pseudo-first-order catalytic constant k_{cat} = 0.09 s^{-1}. Enzymatic efficiency for probe **1**, as estimated by a $k_{cat}/K_m = 4.7 \times 10^4\ M^{-1}\ s^{-1}$, was higher than that of a commercially available reference substrate, 4-methylumbelliferyl phosphate (4-MUP, $k_{cat}/K_m = 2.80 \times 10^4\ M^{-1}\ s^{-1}$) under identical assay conditions.

Next, we investigated the utility of probe **1** for the evaluation of the ALP inhibitor. The inhibition of ALP activity was performed with a well-known ALP-inhibitor,[18] levamisole, at pH 9.0. ALP was pre-incubated with different concentrations of the inhibitor for 10 min at 25 °C. The inhibitor-pretreated ALP-solution was then added to probe **1** in an aqueous solution, and the enzyme-catalyzed hydrolysis reaction was monitored by measuring the fluorescence intensity at 525 nm. As shown in Figure 4, the enhancement of the fluorescence intensity of probe **1** was inhibited in a dose-dependent fashion. In particular, the addition of 75 μM levamisole entirely blocked the increase of fluorescence intensity, confirming that the enzymatic activity of ALP was required to hydrolyze probe **1** and result in fluorescent product **2**. The IC_{50} was determined to be 13.3 μM for levamisole, which is in good agreement with the reported literature value determined by other methods.[19] These results highlight the utilities of probe **1** for both ALP activity assays and the evaluation of inhibitor.

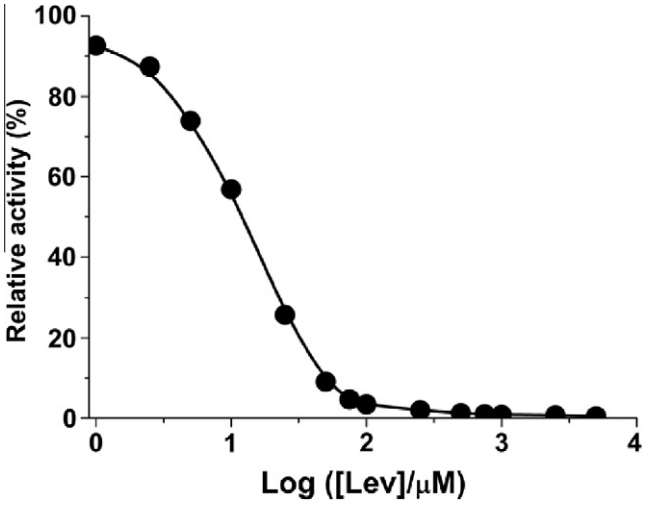

Figure 4. Inhibition assay of ALP activity by levamisole. Relative emission intensity of probe **1** as a function of the concentration of the inhibitor. ALP (50 nM) was incubated with the inhibitor for 10 min at 25 °C before the addition of probe **1** (20 μM). After 10 min incubation at 37 °C, emission intensity at 525 nm was recorded (λ_{ex} 340 nm). Each point was obtained from the average value of three measurements.

In summary, we have developed a new ALP probe based on a unique ESIPT turn-on mechanism. This probe exhibited a large fluorescence response in addition to its excellent sensitivity toward ALP. We anticipate that the probe will accelerate the development and characterization of ALP inhibitors and activators based on in vitro fluorogenic assays, as well as increase the understanding of its biological and pathological roles in living systems.

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Supplementary data

Supplementary data (detailed experimental procedures, additional UV–visible and emission spectra, enzyme assay studies) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.021.

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