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Development of Ratiometric Fluorescent Probes for Phosphatases by Using a pK_a Switching Mechanism

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The use of fluorescent probes in fluorimetric assays is particularly useful in physiological studies because of their high sensitivity and noninvasiveness. For example, the widely used Ca^{2+} probe fura-2[1] and the subsequently developed fluorescent probes[2] have contributed significantly to the rapid progress of intracellular Ca^{2+} signaling studies. One of the outstanding characteristics of fura-2 is that it exhibits a shift in its excitation spectra in response to changes in Ca^{2+} concentration, which enables ratiometric fluorescence measurement at two wavelengths. Ratiometric measurement, in which the fluorescence intensity is monitored at two excitation or two emission wavelengths and the ratio of the two values is calculated, is more practical than normal fluorescence intensity measurement, because ratiometric measurement can exclude such variables as the influence of dye localization and fluctuation of excitation light intensity.[3]

For the rational design of ratiometric fluorescent probes, the resonance energy transfer (RET) mechanism is quite useful.[4] However, small-molecule RET probes generally require complicated synthesis, which involves conjugation of two different fluorescent dyes. Therefore, a new design principle for ratiometric probes is required. In this study, we developed a novel design strategy for ratiometric fluorescent probes. We applied this strategy in the development of fluorescent probes for detecting phosphatase activity.

Phosphatases catalyze the dephosphorylation of various types of biomolecules, including proteins, nucleic acids, and lipids, and play significant roles in the regulation of metabolic pathways in living organisms. Phosphatases are categorized into several groups, including alkaline phosphatases (ALP),[5] acid phosphatases (ACP),[6] serine/threonine phosphatases,[7] and tyrosine phosphatases (PTP)[8] to name a few. Thus far, several fluorescent probes have been developed for detecting phosphatase activity.[9–12] One prototype is 4-methylumbelliferyl phosphate (MUP),[9] which is hydrolyzed by several types of phosphatases, resulting in an increase in the fluorescence intensity. Other fluorescent phosphatase probes such as 3,6-fluorescein diphosphate (FDP)[10] and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)[11] are also widely used. However, these probes do not have ratiometric fluorescence properties. Thus, the development of ratiometric fluorimetric probes that can detect phosphatase activities has been attempted by many research groups.

In addition to the aforementioned limitation, known phosphatase probes have another drawback: they have an aryl phosphate monoester moiety; phosphodiesterases convert them to the corresponding aryl alcohol, which fluoresce more strongly than the phosphate monoesters. Although they are considered structural analogues of phosphotyrosine and are used as fluorescent probes for detecting tyrosine phosphatase activity,[11,12] they are hydrolyzed by serine/threonine phosphatases[13] and ALP/ACP.[13,14] Usually, several types of phosphatase are activated in biological samples. When known phosphatase probes such as DiFMUP are used for the detection of phosphatase activity, the output fluorescence signal represents the sum of the activities of several phosphatases. Therefore, there is a requirement for more specific probes for individual phosphatases. For developing a specific probe for a phosphatase, the design of the enzyme-recognizing structure is important. The known probes always require an aryl phosphate monoester structure, and this structural requirement imposes severe limitations on probe design. Herein we report a novel design strategy for fluorescent probes that have an alkyl phosphate monoester structure. We investigated the specificity of the synthesized probes toward several phosphatases and discuss the correlation between their structure and the kinetic parameters in reaction with ACP.

In our design of new ratiometric probes, we initially focused on the fluorescence properties of coumarins. Coumarins containing an electron-donating substituent at the 6- or 7-position generally fluoresce in aqueous solution and have been extensively used for the fluorescence detection of various enzyme activities.[9,11,13,15] In particular, 7-hydroxycoumarins (umbelliferones) have been widely used because they have strong fluorescence intensities and they are easily synthesized. One of their distinctive characteristics is that their fluorescence properties are affected by solution pH;[16] excitation wavelength maxima (λ_{ex}) are approximately 330 and 370 nm in acidic and alkaline solution, respectively (Figure 1 A). This is because the protonation of the 7-hydroxy group affects fluorescence. By varying the pK_a value of the 7-hydroxy group through judicious substitution, the relative proportion of the phenol and phenolate forms in a neutral buffered solution can be systematically varied. Thus, if the pK_a value of the 7-hydroxy group can be controlled by an enzymatic reaction, the excitation spectrum of coumarin would change in response to the enzyme activity.

The conventional approach to vary the pK_a values of 7-hydroxycoumarins involves substitution of the hydrogen atom at the 6- or 8-position with a halogen atom such as fluorine or chlorine; this substitution decreases the pK_a value by an induc-

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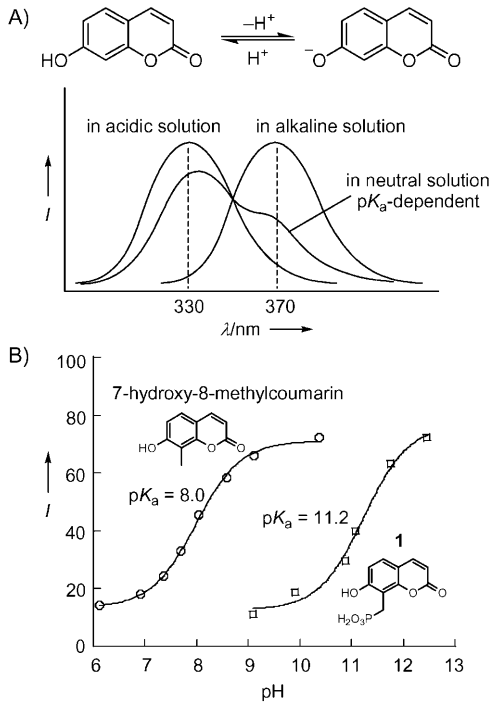
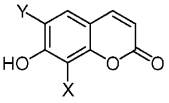


Figure 1. A) pH dependence of the excitation spectra of 7-hydroxycoumarin in aqueous solution (I = fluorescence intensity). B) Effect of a neighboring anionic group on the pK_a values of 7-hydroxycoumarins; fluorescence intensities were measured at λ_{ex} = 380 nm and λ_{em} = 470 nm (25 °C).

tive effect.[17] On the other hand, it has been reported that Calcein Blue (pK_a = 6.9[18]) and other 8-aminomethyl-substituted 7-hydroxycoumarins (pK_a = 6.6–6.7[19]) have lower pK_a values than 7-hydroxy-4-methylcoumarin (pK_a = 7.8[17]). In these cases, the positively charged ammonium groups probably interact with the 7-hydroxy group through hydrogen bonding or electrostatic interactions to enhance deprotonation. By extending this concept, we hypothesized that an anionic group at the 6- or 8-position might increase the pK_a value of the 7-hydroxy group in the opposite manner.



Compound	X	Y
1	$CH_2PO_3H_2$	H
2	$CH_2OPO_3H_2$	H
3a	$CH_2CH_2OPO_3H_2$	H
3b	CH_2CH_2OH	H
4a	H	$CH_2CH_2OPO_3H_2$
4b	H	CH_2CH_2OH
5a	$CH_2CH_2CH_2OPO_3H_2$	H
5b	$CH_2CH_2CH_2OH$	H

To confirm our hypothesis, we synthesized 7-hydroxy-8-phosphorylmethylcoumarin **1** (the synthesis is shown in Scheme S1 in the Supporting Information) and estimated the pK_a value from the fluorescence intensity at various pH values (Figure 1 B). As expected, the pK_a value of **1** was considerably higher (pK_a = 11.2) than that of 7-hydroxy-8-methylcoumarin (pK_a = 8.0). This indicates a strong interaction between the anionic phosphate group and the 7-hydroxy group. We exploited this pK_a switch to develop a new type of fluorescent probe, as shown in Figure 2. Here, an anionic group is introduced in the coumarin scaffold at the 6- or 8-position through a tether to increase the pK_a value of the 7-hydroxy group.

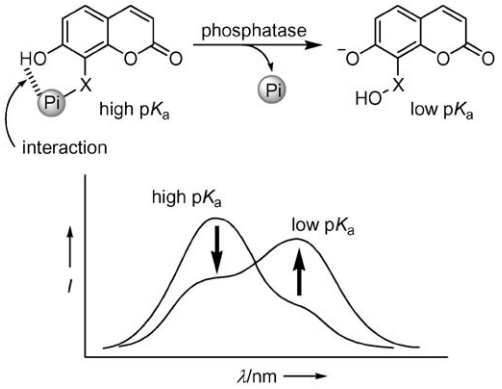


Figure 2. Change in the excitation spectrum of 7-hydroxycoumarin derivatives upon reaction with phosphatases (Pi: phosphate group, X: alkyl linker).

After enzymatic removal of this moiety, the pK_a value would decrease due to loss of the electrostatic interaction, resulting in a change in the excitation spectrum. We selected a phosphate group as the leaving anionic group. In this case, the probe can detect phosphatase activities.

We synthesized 7-hydroxy-8-phosphoryloxymethylcoumarin **2**. However, the compound was unstable and decomposed in aqueous solution. This is probably because the 7-hydroxy group accelerated the removal of the phosphate group to form quinone methide, as reported previously,[16] as phosphate is a good leaving group. We then synthesized **3a**, which has an 8-phosphoryloxyethyl group. We also synthesized **4a** and **5a** in order to examine the effect of the position of the anionic group and the alkyl chain length, respectively. Compounds **3b**–**5b**, lacking phosphate, were synthesized as control compounds. The synthetic routes are shown in Scheme S2 in the Supporting Information.

The pH dependence of the excitation spectrum of **3a** was measured (Figure 3 A). Peaks were observed at 333 nm (pH 4.5) and 381 nm (pH 9.3). Between pH 6.5 and 9.3, the excitation spectrum has an isosbestic point at 350 nm; this indicates that only two species, the protonated and deprotonated forms of the 7-hydroxy group, are present in this pH range. The pK_a values of the 7-hydroxy groups of **3a** and **3b** were calculated to be 8.3 and 7.8, respectively, by curve fitting from plots of fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 470 nm) versus pH (Figure 3 B). As we had hypothesized, **3a** showed a greater pK_a value than **3b**.

Compounds **4a** and **5a** showed similar pK_a values (8.1 and 8.2, respectively). The pK_a values of the corresponding dephosphorylated products **4b** and **5b** were 7.7 and 7.8, respectively. In each case, the pK_a value of the phosphate monoester is greater than that of the corresponding alcohol. On the basis of

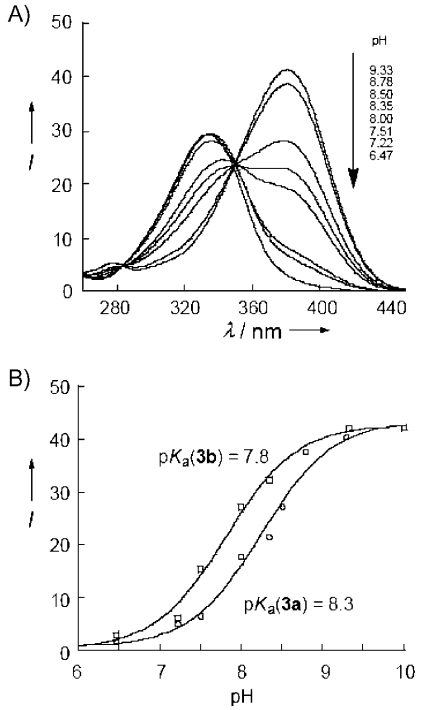


Figure 3. A) Change in the excitation spectrum of **3a** at various pH values (λ_{em} = 470 nm). B) Fluorescence intensities of **3a** (○) and **3b** (□) at various pH values (λ_{ex} = 380 nm, λ_{em} = 470 nm).

these results, it was expected that the excitation spectrum of **3a**–**5a** in neutral buffer solution would change after phosphatase-mediated dephosphorylation, as shown in Figure 2.

We examined the suitability of these compounds as fluorescence probes for phosphatases. When ACP was added to a 10 μM solution of **3a**, the excitation spectrum changed in the expected manner as a function of time. Compounds **4a** and **5a** showed similar spectral changes. For example, the excitation spectrum of **5a** shows a decrease at 330 nm and an increase at 380 nm (Figure 4), originating from the protonated and the deprotonated forms, respectively. The occurrence of the enzyme reaction was confirmed by HPLC (Figure S1, Supporting Information).

Next, we studied the reactivity of other phosphatases toward **3a**. As expected, **3a** was not dephosphorylated by

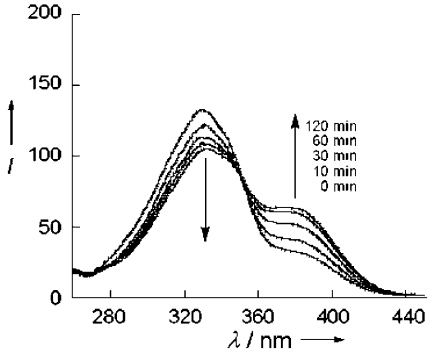


Figure 4. Excitation spectra of **5a** before and after the addition of ACP in 100 mM HEPES buffer solution (pH 7.4) (λ_{em} = 470 nm).

PTPs (CD45 and PTP1B) or by serine/threonine phosphatases (PP1 and $PP2A_1$). On the other hand, ALP induced a partial change in the excitation spectrum of **3a**. However, the reaction rate decreased progressively as the reaction proceeded. This result suggests that the reaction product **3b** inhibits ALP activity. To confirm this putative product inhibition, we examined the effect of **3b** on the fluorescence intensity increase of a commercial fluorescent substrate, DiFMUP. The fluorescence intensity of DiFMUP increased significantly after the addition of ALP, whereas pre-incubation with **3b** inhibited this increase (Figure S2, Supporting Information). This supports the view that **3b** causes product inhibition. This inhibition was not observed with the other phosphatases we studied, so this may provide a clue to the development of selective ALP inhibitors. It is also noteworthy that some coumarin derivatives are protein kinase inhibitors.[17]

To examine the properties of the new fluorescent probes more quantitatively, we estimated the parameters K_M and V_{max} of ACP for **3a**–**5a** by fitting the plot of the initial velocity of the enzyme reaction versus substrate concentration with the Michaelis–Menten equation (Table 1). DiFMUP was used as the

Table 1. Kinetic parameters of **3a**, **4a**, and **5a** with acid phosphatase.[a]

Compound	K_M [μM]	$V_{max} \times 10^9$ [M min^{-1}]	V_{max}/K_M [min^{-1}]
3a	54 ± 18	4.7 ± 0.3	8.7×10^{-5}
4a	107 ± 27	53 ± 3	5.0×10^{-4}
5a	32 ± 12	17 ± 2	5.3×10^{-4}
DiFMUP	41 ± 19	31 ± 9	7.6×10^{-4}

[a] Kinetic data were measured at 30 °C in 100 mM HEPES buffer (pH 7.4) containing 1.0 mM DTT and 1.0 mM EDTA.

standard substrate under the same conditions. Among the three synthesized probes, **4a** and **5a** showed high V_{max}/K_M values, which were similar to that of DiFMUP, whereas **3a** was the worst substrate. The difference in the V_{max}/K_M values of **3a** and **4a** presumably reflects the difference in steric crowding around the phosphate group, because **3a** and **4a** are regioisomers; that is, **3a** is an 8-substituted coumarin and **4a** is a 6-substituted coumarin, and the 6-position of 7-hydroxycoumarin is less crowded than the 8-position. Compound **5a** had an additional methylene group in the linker, and it showed an approximate sixfold increase in the V_{max}/K_M value in comparison with **3a**. This finding indicates that further modification of the linker group might yield more selective and reactive fluorescent probes for ACP. Our design principle could also be used to develop specific probes not only for ACP but for other phosphatases as well.

In conclusion, we have developed a novel design strategy using a pK_a switching mechanism for the design of ratiometric fluorescent probes to detect phosphatase activity. This design strategy is based on a pK_a shift of the 7-hydroxy group of 7-hydroxycoumarin derivatives induced by an adjacent anionic group, which is directly coupled to a change in the excitation spectrum. The synthesized probes are efficiently dephosphorylated by ACP, and changes in the excitation spectrum are ob-

served. These probes are not dephosphorylated by two protein tyrosine phosphatases (PTP1B and CD45) and serine/threonine phosphatases (PP1 and $PP2A_1$), but are slightly dephosphorylated by ALP. The widely used fluorescent phosphatase probe, DiFMUP, responds to all types of phosphatase activity; therefore, our new probes show different enzyme specificity from previously known probes. The enzyme kinetic data indicate that modification of the linker could dramatically change the enzyme affinity.

Our probe design strategy has afforded ACP-selective fluorescent probes. However, in principle, the probe structure can be changed significantly, provided that the hydroxy group (the 7-hydroxy group in this case) interacts with the ionic group (phosphate group in this case). Thus, this strategy should be applicable to the synthesis of fluorescent probes that are highly specific for various types of phosphatases by modifying the linker structure. Further, as discussed above, ratiometric fluorescent probes are preferable for quantitative bio-imaging experiments. As our probe design strategy intrinsically yields ratiometric probes, it should be helpful for the rational development of a wide range of fluorescent probes for the ratiometric bio-imaging of various hydrolases such as phosphodiesterases and sulfatase in living organisms.

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