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A Fluorescent Anion Sensor That Works in Neutral Aqueous Solution for Bioanalytical Application

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Abstract: Anion recognition and anion sensing are of interest because anions play many important roles in living organisms. Most currently known anion sensors work only in organic solution, but sensors for biological applications are required to function in neutral aqueous solution. We have designed and synthesized a novel fluorescent sensor for anions. The sensor molecule **1**-Cd^{II} contains 7-amino-4-trifluoromethylcoumarin as a fluorescent reporter and Cd^{II}-cyclen (1,4,7,10-tetraazacyclododecane) as an anion host. In neutral aqueous solution, Cd^{II} of **1**-Cd^{II} is coordinated by the four nitrogen atoms of cyclen and the aromatic amino group of coumarin. When various anions are added to 100 mM HEPES buffer solution (pH 7.4) containing **1**-Cd^{II}, the aromatic amino group of coumarin is displaced from Cd^{II}, causing a change of the excitation spectrum. While pyrophosphate and citrate were detected with high sensitivity, fluoride and perchlorate produced no response. Among organic anions, ATP and ADP gave strong signals, while cAMP showed little signal. By utilizing the different affinities of the sensor for AMP and cAMP, the activity of phosphodiesterase, which cleaves cyclic nucleotide, was monitored in real-time. The sensor should have many biochemical and analytical applications and the sensing principle should be widely applicable to the sensing of other molecules.

Introduction

Fluorescent sensors are useful to analyze and clarify the roles of biomolecules in living systems; for example, several functions of intracellular Ca^{II} have been elucidated by using fluorescent Ca^{II} indicators.[1] Moreover, many other metal sensors and pH sensors have been developed.[2] There is now increasing interest in anion recognition[3] and anion sensing,[4] because many organic and inorganic anions play important roles in living organisms. Development of anion sensors may also lead to the development of novel sensors that can detect bioorganic molecules containing anionic groups intramolecularly. However, most known anion sensors only function in organic media and it is difficult to use them in biochemical or physiological experiments.

Anions are generally larger than cations such as metal ions, and therefore anions are more subject to solvation than cations. In organic solvents, it is not so difficult to capture and detect anions because the solvation energy is relatively small and electrostatic interactions operate effectively. However, in aqueous solvents, which are relevant to biological applications, it is very difficult to recognize anions because of the strong hydration. So far, only a few fluorescent anion sensors that work in aqueous solution have been developed, although many are known for organic environments.

A fluorescent anion sensor for use in aqueous solution must meet two requirements. One is sufficiently strong affinity for anions in water, and the other is the ability to convert anion recognition into a fluorescence signal. Most known anion sensors do not have a sufficiently strong affinity for anions in water, although they satisfy the latter requirement. Although some anion hosts can capture anions in aqueous solvent, they are only host molecules, not sensor molecules. It is difficult to satisfy both requirements, simultaneously. Czarnik et al. succeeded in

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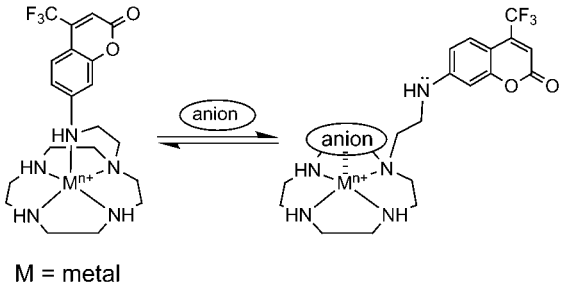


Figure 1. Design concept of sensing anions. M^{n+} is a metal ion that can be chelated stably by cyclen.

the development of fluorescent anion sensors, which work in aqueous solution.[5] The sensing mechanism of these sensors is based on the anion-binding-induced pK_a change of an amino group adjacent to a fluorophore. However, it has been difficult to apply this sensing mechanism to biological experiments, because the sensors have polyamine ligands which chelate metal ions and change the fluorescence properties by chelating them. Although a metal ion insensitive sensor is also reported, it is a sensor for large polyanions such as DNA, not for small molecular anions.[6]

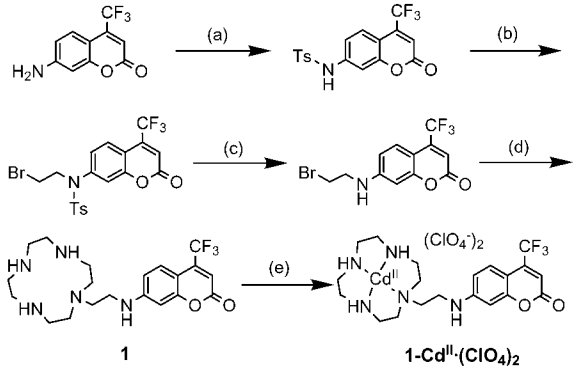
In this paper, we present a novel approach to detect anions. We used a macrocyclic polyamine−metal complex as an anion host, and a fluorescent aromatic amine as a switch and a fluorescent reporter. On the basis of this novel anion sensing mechanism, we developed a practical fluorescent sensor. We show that this new anion sensor can function in neutral aqueous solution even containing 100 mM HEPES buffer. Moreover, to demonstrate the utility of this sensor for bioanalytical applications, we show that this sensor is applicable for real-time sensing of phosphodiesterase activity.

Results and Discussion

Design Concept. First, we have to choose an anion host that can recognize anions in aqueous solution. It is known that the Zn^{II}-cyclen (1,4,7,10-tetraazacyclododecane) complex is a good host molecule for anions in neutral aqueous solution, and anions coordinate to Zn^{II} as the fifth ligand.[7] Therefore, we decided to utilize this complex or an analogous compound. Second, when we use this complex as the anion host, we have to convert anion recognition into a fluorescence signal. We thought that if an intramolecular ligand coordinates to the metal, which is chelated by cyclen, with a relatively weak affinity, an anion species could displace the ligand and this event could trigger an optical signal. So we chose the aromatic amino group of a 7-aminocoumarin as the fifth ligand. As shown in Figure 1, we expected that the coordination of an anion to the metal would cause dissociation of the 7-amino group of 7-amino-4-trifluoromethylcoumarin from the metal and trigger a change in the fluorescence spectra, because functional substitution at the 7-position of the coumarin ring affects the fluorescence spectra of 7-substituted coumarins.[8]

Preparation of Compounds. The cyclen-conjugated coumarin **1** was synthesized in 4 steps from 7-amino-4-trifluoromethylcoumarin (Scheme 1), and purified by NH-silica gel (Fuji Silysia Chemical Ltd.) column chromatography. Complex **1**-Cd^{II}, the Cd^{II} complex of **1**, was obtained as the perchlorate salt and recrystallized from 2-propanol/H_2O. Details of the synthetic procedure are described in Supporting Information.

Scheme 1. Synthetic Route to **1** and **1**-Cd^{II} Complex[a]



[a] Conditions: (a) TsCl, pyridine. (b) $BrCH_2CH_2Br$, Cs_2CO_3. (c) Concentrated H_2SO_4, 90 °C. (d) Cyclen. (e) $Cd(ClO_4)_2$, 60 °C.

Absorption and Excitation Spectra of 1−Metal Complexes. We measured the absorption and fluorescence spectra of **1** with Zn^{II}, Cd^{II}, Cu^{II}, and no metal to find which metal would fit best with our design concept. These metals are well-known to be strongly coordinated by cyclen.[9] The desired spectral change was a blue shift of the absorption and the excitation spectra, because coordination of the 7-amino group to the metal is required for anion sensing, as shown in Figure 1, and the participation of nitrogen lone pairs in coordinating the metal would induce such a wavelength shift.

Absorption and excitation spectra of **1** with Zn^{II}, Cd^{II}, Cu^{II}, and no metal are shown in Figure 2. We measured these spectra after sufficient equilibration (more than 3 h), because formation of these complexes was very slow. The absorption spectral peak of **1** was at 382 nm, and those of **1** + Zn^{II}, **1** + Cd^{II}, and **1** + Cu^{II} were at 388, 342, and 342 nm, respectively. When we added Cd^{II} and Cu^{II} to a solution of **1**, a blue shift of each absorption spectrum was observed. This supports the idea that the 7-amino group coordinates Cd^{II} and Cu^{II}. The absorption spectrum of **1** + Zn^{II} was slightly red-shifted. Although this red shift shows that there is some interaction between Zn^{II} and 7-amino-4-trifluoromethylcoumarin, the 7-amino group probably does not coordinate to Zn^{II}, for the reason mentioned above.

The excitation spectra of **1**, **1** + Zn^{II}, and **1** + Cd^{II} resembled their absorption spectra, although the fluorescence intensity of **1** + Zn^{II} increased a little. The fluorescence of **1** + Cu^{II} decreased markedly because of the nature of Cu^{II}; it is generally known that Cu^{II} ion quenches the fluorescence of various fluorescent compounds.[10]

From the principle of anion sensing shown in Figure 1, the central metal ion needs to be coordinated by five nitrogen atoms including the 7-amino group. Therefore, if Zn^{II} ion is not coordinated by the 7-amino group of **1** in neutral aqueous solution containing 100 mM HEPES, **1**-Zn^{II} complex is unable to act as an anion sensor. Moreover, because the fluorescence of the **1**-Cu^{II} complex was strongly quenched, **1**-Cu^{II} is also not suitable for a fluorescence sensor. Thus, based on the

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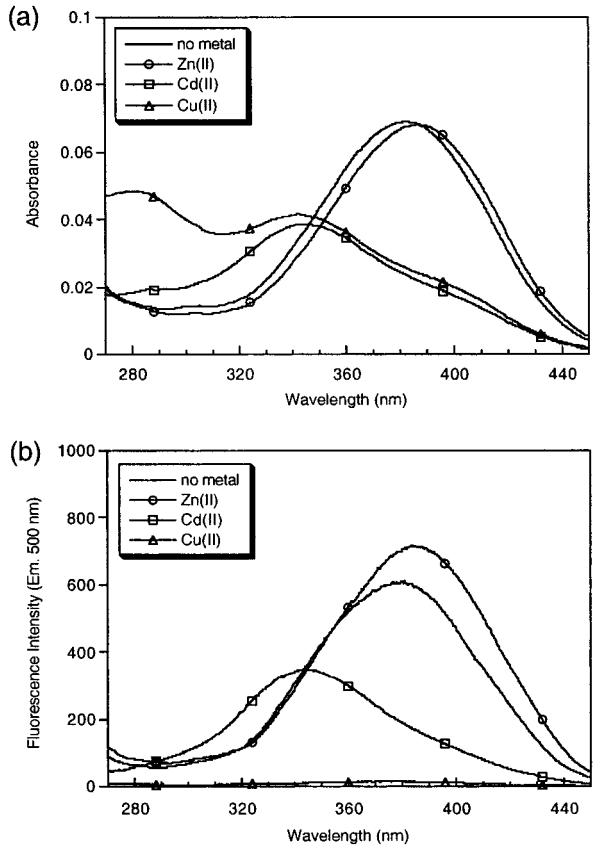

Figure 2. (a) Absorption spectra and (b) excitation spectra (emission wavelength is 500 nm) of **1** with various metals. The concentration of **1** was 5 μM. All metals were added at 1.2 equiv with respect to **1**. Solutions were buffered with 100 mM HEPES (pH 7.4).

Table 1. Stability Constants log β_{pqr} for $M_pL_qH_r$ Systems ($M = Zn^{II}$, Cd^{II}, $L = \mathbf{1}$)[a]

M	p	q	r	log β_{pqr}
Cd^{II}	1	1	0	11.38 ± 0.05
	1	1	−1	4.46 ± 0.06
	1	1	−2	-3.95 ± 0.08
Zn^{II}	1	1	0	10.80 ± 0.08
	1	1	−1	3.87 ± 0.09
	1	1	−2	4.61 ± 0.12
H^+	0	1	1	9.67 ± 0.01
	0	1	2	18.06 ± 0.01

[a] $\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$. Conditions: 0.1 M Et_4NClO_4 ($I = 0.1$), 25 °C. [L] = 0.5 mM, 0.17 mM.

absorption and excitation spectra, we concluded that **1**-Cd^{II} would be the best fluorescent anion sensor based on our design principles, as shown in Figure 1.

Potentiometric pH Titration. Next, we carried out potentiometric pH titration experiments. We measured the pH titration curves of **1**·4$HClO_4$, **1**·4$HClO_4$ + $Zn(ClO_4)_2$, and **1**·4$HClO_4$ + $Cd(ClO_4)_2$, and calculated the ligand protonation constants and the metal complexation constants from these data by using a computer program. The results are shown in Table 1. The protonation equilibrium constants of **1** were 9.67 (log K_1 = log β_{011}) and 8.39 (log K_2 = log β_{012} − log β_{011}). The other lower pK_a values could not be calculated.

The stability constants of **1** for Zn^{II} and Cd^{II}, which are shown as log β_{110} in Table 1, were calculated; the log K(**1**-Zn^{II}) and log K(**1**-Cd^{II}) values are 10.80 and 11.38, respectively. The reported log K(cyclen-Zn^{II}) and log K(cyclen-Cd^{II}) are 16.2 and 14.3, respectively.[11] If the coordination forms of **1**−metals are the same as those of cyclen, log K(**1**-Zn^{II}) might be expected to be larger than log K(**1**-Cd^{II}), but this is not the case; namely, the order of relative stability of the **1**−metal complexes was reversed as compared to the cyclen−metal complexes. This result suggests that the coordination form of the **1**-Cd^{II} complex is different from that of the **1**-Zn^{II} complex. We consider that Zn^{II} is not coordinated by the aromatic amino group: water molecules may prevent the aromatic amino group from coordinating to Zn^{II} even in the absence of anions that bind Zn^{II} strongly. On the other hand, the results are consistent with the idea that Cd^{II} is chelated by five nitrogen atoms, i.e., the four amino groups of cyclen and the aromatic amino group. Thus, the pH titration results also suggest that the **1**-Cd^{II} complex meet the design principle in Figure 1.

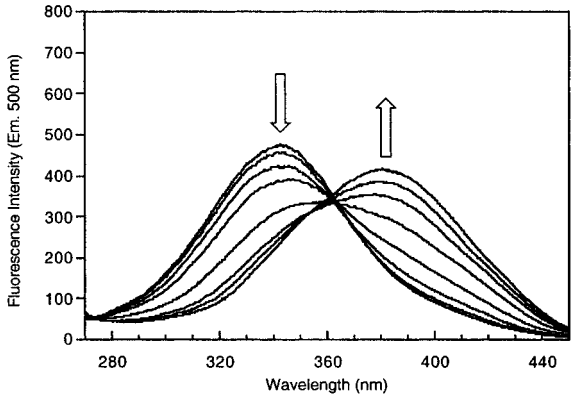

Figure 3. Excitation spectra of 5 μM **1**-Cd^{II} upon addition of sodium pyrophosphate (0, 0.003, 0.01, 0.03, 0.1, 0.3, 3, 10 mM) in 100 mM HEPES buffer (pH 7.4) at 25 °C. The emission wavelength was 500 nm.

Fluorescence Detection of Anions with 1-Cd^{II}. We added various anions to a solution of synthesized **1**-Cd^{II} (5 μM) to investigate what effects various anions have on the excitation spectra. All spectra were measured in the presence of 100 mM HEPES buffer (pH 7.4) at 25 °C.

When sodium pyrophosphate (PPi) solution was added to a solution of **1**-Cd^{II}, the excitation spectrum shifted dose-dependently toward longer wavelength. As shown in Figure 3, the λ_{max} shifted from 342 to 383 nm after addition of 10 mM PPi. The excitation spectrum of **1**-Cd^{II} + 10 mM PPi resembled that of the free base **1** (Figure 2); for instance, both peak wavelengths were about 380 nm. As described in the Design Concept section, in the case of 7-aminocoumarins, a bathochromic shift of the excitation spectrum indicates that the electron density of the 7-amino group has increased.[8] These results indicate that the labile fifth ligand (the aromatic amino group) was dose-dependently replaced by PPi anion, as we expected (Figure 1). In short, in neutral aqueous solution, PPi has a larger affinity for the Cd^{II}-cyclen complex than the aromatic amino group has, even though the amino group is located intramolecularly.

Next, we examined the effects of other anions. Some other anions caused spectral changes of **1**-Cd^{II} and the apparent dissociation constant (K_d) values are shown in Table 2. These K_d values were calculated by fitting the fluorescence intensity changes as described in the Experimental Section. As we expected, citrate showed strong affinity. Phosphate had a much

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Table 2. Apparent Dissociation Constants (K_d) of Sensor **1**-Cd^{II} for Anions in 100 mM HEPES Buffer (pH 7.4)[a]

anion	K_d (M)	anion	K_d (M)
pyrophosphate	7.5×10^{-5}	ATP	1.4×10^{-5}
citrate	9.0×10^{-5}	ADP	2.6×10^{-5}
I^-	9.2×10^{-3}	GMP	4.8×10^{-5} [c]
phosphate	1.5×10^{-2}	AMP	4.4×10^{-4}
Br^-	3.2×10^{-2}	UMP	1.7×10^{-3}
Cl^-	9.0×10^{-2}	CMP	1.9×10^{-3}
F^-	*b*	cAMP	*b*
ClO_4^-	*b*		

[a] All anions were added as sodium or potassium salts. All K_d values were calculated under the condition with 100 mM HEPES buffer (pH 7.4). [b] K_d was too large to be calculated. [c] This value is smaller than the real K_d, because dynamic quenching was observed besides static quenching.

weaker affinity than PPi, probably because the protonated forms, HPO_4^{2-} and $H_2PO_4^-$, are dominant species in aqueous solution of pH 7.4 and they have smaller numbers of negative charges than PPi. Perchlorate did not cause any change even at 100 mM. With regard to oxoacids, multivalent anions bound to **1**-Cd^{II} more strongly than mono- or divalent anions. These data suggest that anions interact with **1**-Cd^{II} through multipoint recognition, probably with both the metal and NH protons of the cyclen ring.

For halogen ions, the spectral changes occurred in the order $I^- > Br^- > Cl^- \gg F^-$, reflecting the degree of soft basicity. This is consistent with the fact that Cd^{II} is a soft acid and has a high affinity for soft bases.

We next examined some nucleotides, because they are negatively charged organic compounds with key roles in living organism, especially cyclic nucleotides such as cAMP and cGMP work as intracellular second messengers. Therefore, sensing of various nucleotides is very important. First, four nucleoside monophosphates, AMP, GMP, CMP, and UMP, were added to a solution of 5 μM **1**-Cd^{II}. Interestingly, only GMP quenched the fluorescence and the other three nucleotides caused a red shift of the excitation spectrum, like inorganic anions (Figure 4a). The quenching mechanism of the guanine nucleotide is probably based on photoinduced electron transfer. The apparent dissociation constants are shown in Table 2. These results suggest that both the shape of the excitation spectrum and the dissociation constants are dependent on the type of nucleobase. One possible explanation is that coumarin interacts directly with the base of nucleotides.

Although ADP and ATP had higher affinities for **1**-Cd^{II} than AMP, there was no large difference about binding strength between ADP and ATP. Two cyclic nucleotides, cAMP and cGMP, had lower affinities for **1**-Cd^{II} than their hydrolysis products, AMP and GMP, respectively. The spectral data for cAMP and AMP are shown in Figure 4b. When even 10 mM cAMP was added, the excitation spectral change of **1**-Cd^{II} was small. This spectrum closely resembled the spectrum upon addition of 100 μM AMP. Addition of less than 1 mM cAMP scarcely changed the excitation spectrum of **1**-Cd^{II}. Considering these results, the difference in binding strength between AMP and cAMP was probably based on the number of anionic sites.

Reversibility of Anion Sensing. We examined the reversibility of anion sensing. If the sensing system is reversible, depletion of the anion that coordinates Cd^{II} must produce a blue shift of the excitation spectrum, causing it to revert to the original spectrum. An excess amount of Mg^{II} was added to this

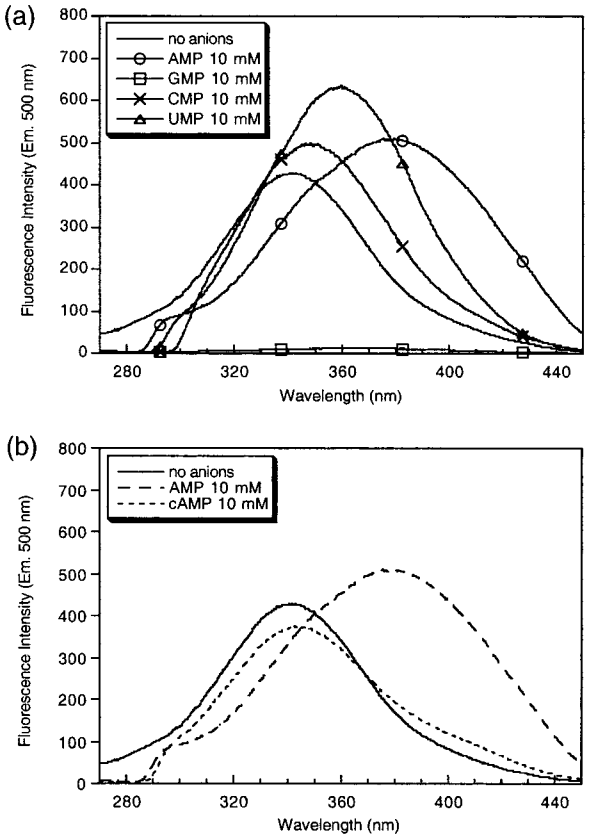

Figure 4. Comparison of the excitation spectra of 5 μM **1**-Cd^{II} upon addition of (a) AMP, GMP, CMP, and UMP and (b) AMP and cAMP. The concentration of each nucleotide was 10 mM.

PPi-containing solution to bind PPi and prevent PPi from coordinating Cd^{II}. When 10 mM $Mg(ClO_4)_2$ was added to a solution of **1**-Cd^{II} containing 1 mM PPi, the excitation spectrum was indeed blue-shifted and the λ_{max} was at 345 nm (Figure 5a). This spectrum almost exactly coincided with the spectrum of **1**-Cd^{II} containing no PPi.

Next, we examined the effect of hydrolyzing pyrophosphate. Inorganic pyrophosphatase is known to convert one pyrophosphate ion to two phosphate ions.[12] Because phosphate coordinates **1**-Cd^{II} more weakly than does PPi, cleavage of PPi should induce a spectral change similar to that seen with a large amount of Mg^{II} ion. When 0.33 unit/mL of inorganic pyrophosphatase and 0.5 mM $Mg(ClO_4)_2$ were added to **1**-Cd^{II} solution containing 1 mM pyrophosphate at pH 7.4, the peak of the excitation spectrum quickly shifted toward short wavelength (Figure 5b). These results indicate that this sensing mechanism is reversible.

Phosphodiesterase Assay. To examine the potential of this system for biochemical applications, we tried to monitor the activity of phosphodiesterase (PDE). We used phosphodiesterase 3′:5′-cyclic mononucleotide,[13] which catalyzes the conversion of cyclic nucleotide to nucleoside monophosphate by cleavage of the phosphodiester of the cyclic nucleotide, e.g., converting cAMP to AMP (Scheme 2). Such enzymes participate in

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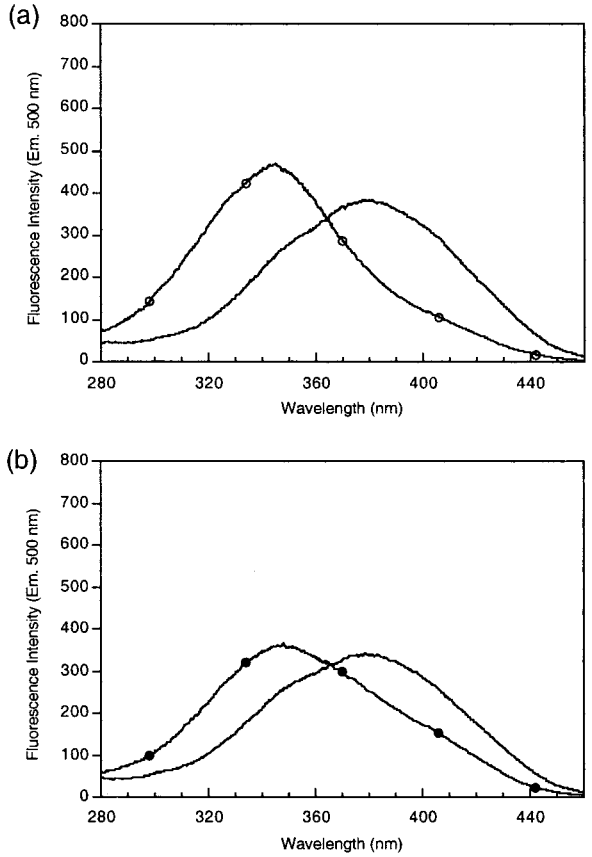
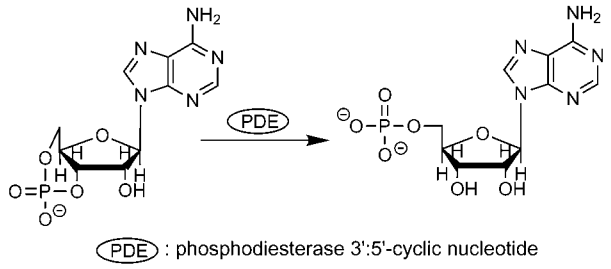


Figure 5. (a) Excitation spectra of **1**-Cd^{II} with 1 mM $Na_4P_2O_7$ (no symbol) and 1 mM $Na_4P_2O_7$ + 10 mM $Mg(ClO_4)_2$ (open circle). (b) Excitation spectra of **1**-Cd^{II} with 1 mM $Na_4P_2O_7$ before (no symbol) and 20 min after (closed circle) addition of inorganic pyrophosphatase. The concentration of **1**-Cd^{II} was 5 μM. The emission wavelength was 500 nm.

Scheme 2



intracellular signal transduction, so the development of sensitive detection systems for their activity[14] is very important.

The developed anion sensor **1**-Cd^{II} recognized AMP much more strongly than cAMP. Therefore, we tried real-time detection of PDE's activity by monitoring the increase of AMP. We measured the excitation spectrum (emission wavelength/500 nm) several times after addition of 2 U/mL (final concentration) of PDE to 5 μM **1**-Cd^{II} solution containing 100 mM HEPES, 10 mM cAMP, 10 μM $CaCl_2$, and 0.02 U/mL of PDE activator at pH 7.4. As shown in Figure 6a,b, the fluorescence intensity (excitation wavelength, 380 nm/emission wavelength, 500 nm) gradually increased. This spectral change shows the conversion from cAMP to AMP. These results show **1**-Cd^{II} is useful for real-time fluorescence assay of phosphodiesterase

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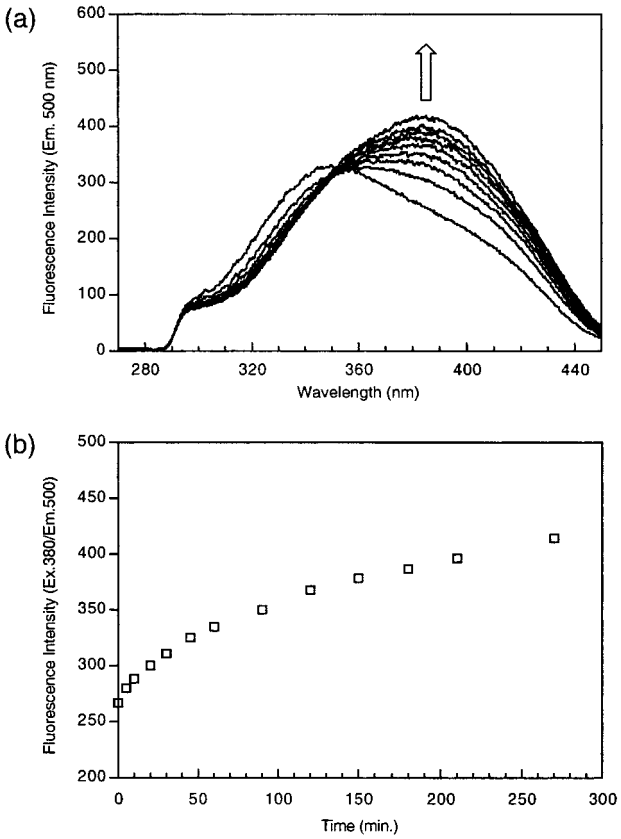

Figure 6. Phosphodiesterase activity assay. (a) Change of the excitation spectra of **1**-Cd^{II}. The emission wavelength was 500 nm. (b) Fluorescence intensity vs time plot. The excitation/emission wavelengths were 380 nm/500 nm.

3′:5′-cyclic nucleotide activity. This approach is expected to be applicable to the assay of other enzyme activities.

Summary and Conclusion

In conclusion, we have developed a novel fluorescent sensor for anions, which works in neutral aqueous solution. The sensing principle is novel and based on coordination chemistry. The sensor **1**-Cd^{II} has a Cd^{II} ion as the central metal of the anion host region, and Cd^{II} is critical to convert anion recognition to a fluorescence signal. When we used Zn^{II} and Cu^{II} instead of Cd^{II}, we could not obtain desirable characteristics for anion sensing. On the basis of spectral and pH potentiometric data, we concluded that Cd^{II} was effective because it is weakly coordinated by the 7-amino group of coumarin, so that some anions could displace it and coordinate to the Cd^{II} ion in its place. We confirmed that this sensing mechanism was reversible. We proved that the activity of phosphodiesterase, which hydrolyzes the phosphodiester bond of cyclic nucleotides, could be monitored by using **1**-Cd^{II}. No other known sensor has this characteristic, so **1**-Cd^{II} could become the preferred anion sensor in many biological and analytical applications. The same sensing principle should also be widely applicable to the sensing of other molecules.

Experimental Section

Measurement of Absorption and Excitation Spectra. All spectra of **1** or **1**-Cd^{II} were measured at 5 μM concentration and 25 °C, using a UV-1600 UV−visible spectrometer (Shimadzu, Kyoto, Japan). The fluorescence spectrometer was an F-4500 (Hitachi, Tokyo, Japan).

Determination of Apparent Dissociation Constants (K_d) of Sensor 1-Cd^{II} for Several Anions. The final concentration of compound **1**-Cd^{II} was 5 μM. All the solutions contained 100 mM HEPES buffer (pH 7.4). Fluorescence values at the peak of the excitation spectra were plotted against the concentration of anions and fitted to the following equation:

$$F = (F_0 + F_{max}[A]/K_d)/(1 + [A]/K_d)$$

where F_0 is the initial F value without anions, F_{max} is the maximum F value, and [A] is the final concentration of the anion added to the solution.

Potentiometric pH Titration. The potentiometric pH titration experiments were carried out with an ionic strength, I, of 0.1 M (Et_4NClO_4) at 25 °C. We conducted titration experiments at 0.17 mM and 0.5 mM 1:1 metal−**1** systems. The detailed procedure has been described elsewhere.[15] A ligand protonation constant, metal complexation constants, and distribution diagrams were calculated with use of the computer programs SUPERQUAD[16] and MINIQUAD.[17]

Phosphodiesterase Assay. The reaction solution contained 100 mM HEPES (pH 7.4), 10 mM adenosine 3′:5′-cyclic monophosphate (cAMP), 10 μM $CaCl_2$, 5 μM **1**-Cd^{II}, 2 U/mL of phosphodiesterase 3′:5′-cyclic nucleotide activator, and 0.02 U/mL of phosphodiesterase 3′:5′-cyclic nucleotide (PDE). The total volume was 3 mL. Excitation spectra were measured with emission at 500 nm. The reaction temperature was 30 °C.

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Supporting Information Available: Supplemental procedures for materials and the synthetic procedure of **1** and **1**-Cd^{II} and characterization data for the synthetic compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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