Real-time Off/On-mode Fluorescence Assay for Enzyme Reactions Involving Nucleoside Polyphosphates by Use of a Xanthene Zn^{II}–Dpa Chemosensor

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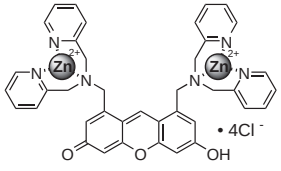
New real-time fluorescent assay systems using a zinc(II) complex as a chemosensor for two enzyme reactions catalyzed by Nudix hydrolases and glycosyltransferases have been developed.

Artificial fluorescent chemosensors for selective detection of the phosphate derivatives of biological significance are a promising molecular tool to analyze their roles and the related biological processes in living systems. To date, a variety of artificial chemosensors for phosphate derivatives have been developed, however, their application to biological assays is still limited, mainly owing to the rather weak binding affinities in aqueous solution as well as the low sensing selectivity for biologically relevant phosphate derivatives.[1] Recently, we have reported the binuclear Zn^{II}–Dpa (2,2′-dipicolylamine) chemosensor **1**–$2Zn^{II}$ (Chart 1) as a selective fluorescent chemosensor for polyphosphate derivatives, which works effectively under neutral aqueous conditions.[2] We describe herein new fluorescence assay systems using **1**–$2Zn^{II}$ for two enzyme reactions, which involve the hydrolysis of diadenosine polyphosphate (Ap_nA) catalyzed by phosphodiesterase and the oligosaccharide synthesis catalyzed by glycosyltransferase. Both assay systems efficiently harness the selective sensing ability of **1**–$2Zn^{II}$ for the polyphosphate derivatives, which enables us to conveniently evaluate the enzyme activities in a real-time manner without the need for chemical labeling of the substrates.



1-$2Zn^{II}$

Chart 1.

As shown in Figure 1, the chemosensor **1**–$2Zn^{II}$ displayed an off/on-type large fluorescence enhancement ($F_{max}/F_{int} > 30$) upon binding to polyphosphate species such as ATP and inorganic pyrophosphate (PPi) with strong binding affinities ($K_{app} > 1 \times 10^6\ M^{-1}$) under neutral aqueous conditions.[2] In contrast, **1**–$2Zn^{II}$ scarcely senses nucleoside polyphosphate lacking a phosphomonoester moiety such as diadenosine tetraphosphate (Ap_4A) and UDP–galactose, and nucleoside monophosphate such as AMP. Taking advantage of this selectivity, we applied **1**–$2Zn^{II}$ to the fluorescence enzyme assay for the hydrolysis of Ap_nA. Ap_nA ($n = 2$–7) is known as an important class of extracellular signaling molecule in a broad variety of tissues.[3] They were hydrolyzed by a series of enzymes known as Nudix hydrolases to release nucleoside 5′-monophosphate.[4] Conventionally, the activities of Nudix hydrolases have been evaluated by HPLC analysis with detection of the nucleoside phosphates produced in enzyme reactions.[5] However, a convenient luminescent method that allows a real-time trace of the reaction has not yet been developed. As a proof-of-principle study, we employed phosphodiesterase IV (PDE) isolated from snake venom, which catalyzes the degradation from Ap_4A to ATP and AMP (eq 1) and the subsequent process from ATP to AMP and PPi (eq 2).[6] Since **1**–$2Zn^{II}$ fluorescently sense ATP and PPi but not Ap_4A and AMP (Figure 1), one can readily expect that the degradation of Ap_4A can be monitored by a fluorescence increase in response to the formation of ATP and PPi. When PDE was added to the assay solution containing 15 μM of Ap_4A and 1 μM of **1**–$2Zn^{II}$ (25 mM HEPES, 5 mM $MgCl_2$, pH 7.75), a large fluorescence enhancement was observed in a time-dependent manner (Figure 2a). The fluorescence enhancement rate (F/F_o) maximally reaches nearly 25-fold. The degradation of Ap_4A and the concomitant formation of AMP and ATP were confirmed by HPLC analysis, showing that the fluorescence enhancement is well correlated with the amount of ATP and PPi produced in the sequential enzyme process (data not shown). As shown in Figure 2b, the initial rate of the fluorescence enhancement (ΔF, min^{-1}) is proportional to the amount of PDE used in the reaction. These results clearly demonstrated that **1**–$2Zn^{II}$ can successfully detect the degradation of Ap_4A in a real-time manner.

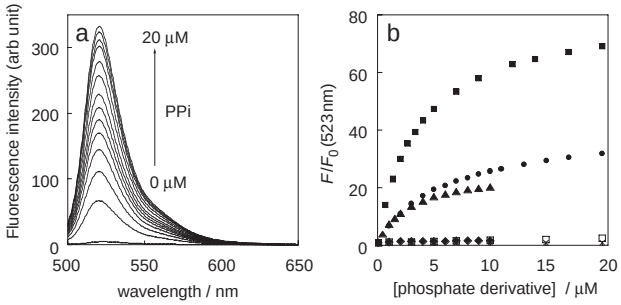


Figure 1. (a) Fluorescence response of **1**–$2Zn^{II}$ upon addition of pyrophosphate (PPi, 0–20 μM). (b) Fluorescence titration profiles of **1**–$2Zn^{II}$ for ATP (●), PPi (■), UDP (▲), Ap_4A (□), AMP (×), and UDP–Gal (◆). Measurement conditions: 1 μM of **1**–$2Zn^{II}$, 25 mM HEPES, 5 mM $MgCl_2$, pH 7.75 for ATP, PPi, Ap_4A, AMP, and 50 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$ for UDP and UDP–Gal, $\lambda_{ex} = 488$ nm.

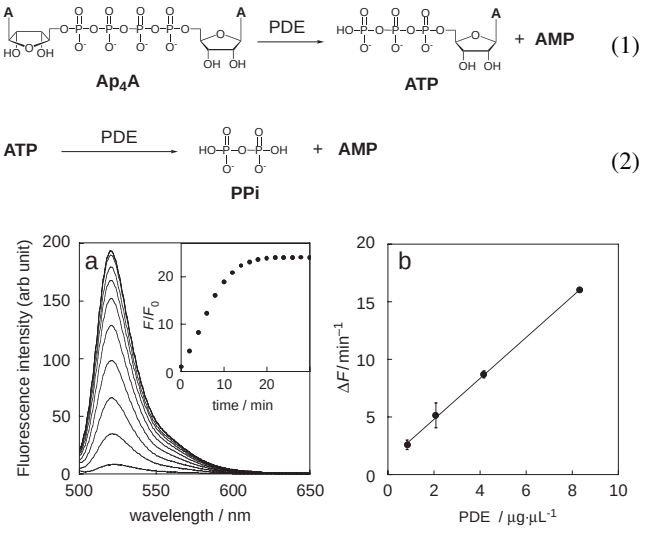


Figure 2. (upper equations) Hydrolysis pathway of diadenosine tetraphosphate (Ap_4A) catalyzed by phosphodiesterase IV (PDE). (a) Fluorescence spectral change of **1**–$2Zn^{II}$ during the degradation of Ap_4A from 0 to 30 min. (inset) Time-trace plot of the fluorescence intensity ($\lambda_{em} = 523$ nm) of **1**–$2Zn^{II}$. Assay conditions: 25 mM HEPES, 5 mM $MgCl_2$, pH 7.75, 37 °C, $\lambda_{ex} = 488$ nm. (b) Plot of the initial rate of the fluorescence increase (ΔF, min^{-1}) as a function of the amount of PDE.

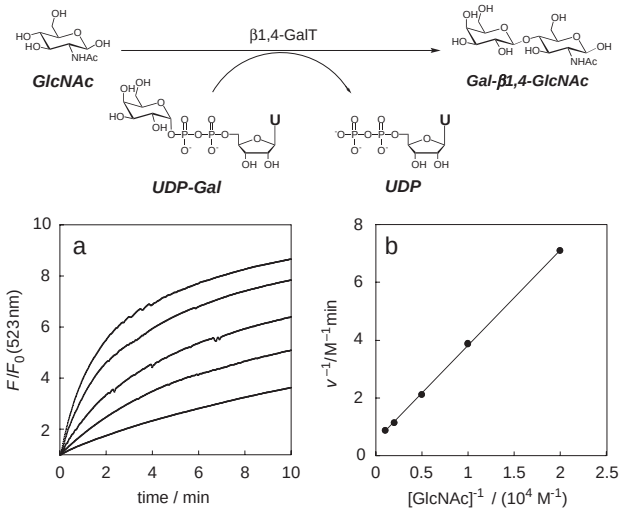


Figure 3. (upper scheme) Glycosyl-transfer reaction catalyzed by β-1,4-galactosyltransferase (β-1,4-GalT). (a) Time-trace plot of the fluorescence intensity ($\lambda_{em} = 523$ nm) of **1**–$2Zn^{II}$ in the glycosyl-transfer reaction between UDP–Gal (20 μM) and GlcNAc (0.05, 0.1, 0.2, 0.5, and 1 mM, from the lowest to the top trace). Assay conditions: 12 mU β-1,4-GalT, 50 mM HEPES, 10 mM NaCl, 0.1 mM $MgCl_2$, 24 μM $ZnCl_2$, pH 7.2, 25 °C, $\lambda_{ex} = 488$ nm. (b) Lineweaber–Burk plot analysis of the glycosyl-transfer reaction shown in Figure 3a.

In a similar strategy, we carried out the real-time fluorescence monitoring for the glycosyl-transfer reaction using **1**–$2Zn^{II}$. Glycosyltransferases, a superfamily of enzymes in biosynthesis of oligosaccharide, play important roles in various cellular events.[7] In the glycosyl-transfer process, a nucleoside sugar such as UDP–galactose (UDP–Gal) is employed as a universal glycosyl donor to form a new glycosyl bond with a variety of acceptor sugars. Since the nucleotide sugar is converted into a nucleoside polyphosphate such as UDP during the glycosyl-transfer reaction, it can be assumed that the selective fluorescence detection of the UDP formation is equivalent to monitoring the glycosyl-transfer reaction. As shown in Figure 1, **1**–$2Zn^{II}$ displays a large fluorescence enhancement upon binding to UDP ($F_{max}/F_{int} = 20$), whereas negligible fluorescence change is detected with UDP–Gal. This selective sensing property is again suitable for the fluorescence assay of the glycosyl-transfer reaction. Based on this principle, we performed the fluorescence monitoring of the glycosyl-transfer reaction between *N*-acetyl glucosamine (GlcNAc) and UDP–Gal catalyzed by β-1,4-galactosyltransferase (β-1,4-GalT) in the presence of **1**–$2Zn^{II}$. As shown in Figure 3a, the large fluorescence enhancement is observed in a time-dependent manner during the reaction. The formation of disaccharide Gal–β-1,4-GlcNAc was confirmed by MALDI-TOF mass analysis after converting it to the corresponding pyridylamino derivative (data not shown). The initial rate of the fluorescence increase depends on the concentration of GlcNAc (Figure 3b). The kinetic analysis using the Lineweaber–Burk plot gives the Michaelis constant for GlcNAc as 0.66 mM, comparable with the value previously reported (1.3 mM).[8] The assay was also applicable to the trisaccharide formation using a disaccharide chitobiose (GlcNAc–β-1,4-GlcNAc) as an acceptor substrate, in which the Michaelis constant for chitobiose was determined to be 0.11 mM (data not shown). Although we previously reported a similar assay system using an anthracene-type Dpa–Zn^{II} complex chemosensor, the fluorescence enhancement (F/F_0) was only less than 2-fold in the detection of the glycosyl-transfer reactions.[9] Compared to the previous system, the present one apparently benefits from the off/on-type larger fluorescence signal change of **1**–$2Zn^{II}$, which should allow more precise and sensitive detection of a variety of glycosyltransferase activities. We believe that the present real-time fluorescence assay system has a significant advantage in its convenience over the conventional methods using radiolabeling or enzyme reaction-coupling techniques.[10]

In conclusion, we have successfully demonstrated the utility of the chemosensor **1**–$2Zn^{II}$ in the fluorescence assay systems for two enzyme reactions. These label-free and real-time fluorescent assays should be useful in general for probing the enzyme activities of a wide variety of Nudix hydrolases and glycosyltransferases, as well as for screening of potent inhibitors.

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