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# Fluorescent Assay of Cyclic Nucleotide Phosphodiesterase Activity in a Neutral Aqueous Solution

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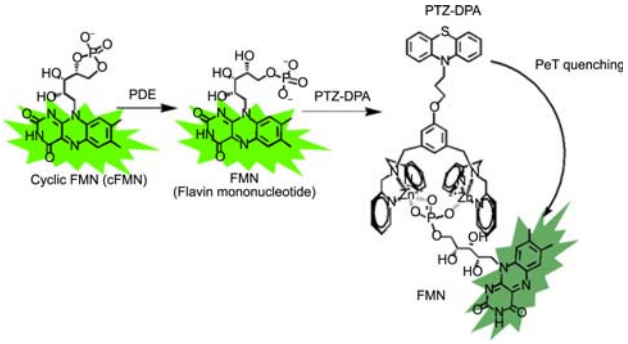
Cyclic nucleotide phosphodiesterase (PDE) hydrolyzes the intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to adenosine monophosphate (AMP) and guanosine monophosphate (GMP).[1] Eleven families of PDE have been reported in mammals[2] and affect cellular activity in the brain, heart, lungs, and muscles.[3] Selective inhibitors of the different PDE isozymes have great potential to be used as novel therapeutic agents in areas such as hypertension, congestive heart failure, thrombosis, glaucoma, and asthma.[4] Among them, the PDE3 inhibitor is used in the treatment of acute heart failure and the PDE5 inhibitor is used to treat erectile dysfunction.

As the inhibition of PDE activity is clinically important, significant effort has been invested in real-time analysis of the PDE enzyme reaction.[5] The radiometric method[6] and HPLC have been used for the assay of PDE. However, the radiometric method is laborious in practice because it requires an isotopically labeled substrate and selectivity of adsorption on the resin, and HPLC analysis does not enable real-time monitoring. Therefore, a fluorescence assay is necessary for the real-time monitoring of enzymatic reactions. Until now, the reported fluorometric detection methods of PDE activity[7] have been used only for the hydrolysis of cAMP[8] and cGMP.[9] Herein we report a chemosensor-based fluorescent real-time assay of PDEs that hydrolyze riboflavin 4',5'-cyclic phosphate (cyclic FMN = cFMN) to flavin mononucleotide (FMN) in a neutral aqueous solution.

The identification of new biological pathways is significant and challenging.[10] Several fluorescent chemosensors[11] have been developed to probe or uncover new biological reactions, which would not have been possible with HPLC or other bioanalytical methods. Recently, several research groups have discovered new biological pathways related to cFMN.[12] cFMN is known as a product of an enzyme reaction, FAD (flavin adenine dinucleotide) cFMN + AMP catalyzed by FMN cyclase.[13] Cameselle and coworkers reported fluorometric HPLC detection of endogenous cFMN in rat liver.[14] However, no assay has been developed to detect FMN produced by the enzymatic reaction of cFMN catalyzed by PDE.

In this paper, we report the first real-time fluorescent monitoring of the hydrolysis reaction of cFMN catalyzed by PDE using the chemosensor **PTZ-DPA** (phenothiazine-Zn (II)·dipicolylamine complex), which subsequently enables the label-free fluorescent real-time assay of PDE.[15] **PTZ-DPA** consists of phenothiazine and bis($Zn^{2+}$-dipicolyl amine), which binds strongly to the phosphate of FMN (Scheme 1). The isoalloxazine ring of FMN emits green fluorescence (quantum yield: 0.26, excitation wavelength: 445 nm, emission wavelength: 525 nm) in water.[16] The fluorescence emission inherent in FMN due to the isoalloxazine moiety is effectively quenched upon binding of the phosphate group of FMN to **PTZ-DPA** because the phenothiazine group of **PTZ-DPA** acts as an electron donor and quenches the emission from the isoalloxazine moiety in the complex by photoinduced electron transfer (PET).[17] We were able to conduct fluorescence monitoring of PDE type IV and type VI activity in a neutral aqueous solution at 25 ºC by following the decrease in the fluorescence intensity of FMN. However, the substrate (cFMN) emits its original fluorescence, as the cyclic phosphate moiety of cFMN is extremely weakly bound to **PTZ-DPA**.




**Scheme 1.** Hydrolysis of cFMN to FMN by **PDE** and FMN fluorescence quenching mechanism by photoinduced electron transfer (PET) of **PTZ-DPA**.

**PTZ-DPA** was synthesized according to the reported method.[15] The PDE activity was investigated by fluorescent time-course measurement assay. To the pH 7.2 HEPES (10 mM) buffer solution containing $MgCl_2$ (1 mM), cFMN (10 μM), and **PTZ-DPA** (50 μM), PDE type IV and type VI were added at 25 ºC in different concentrations (0.25 mg/

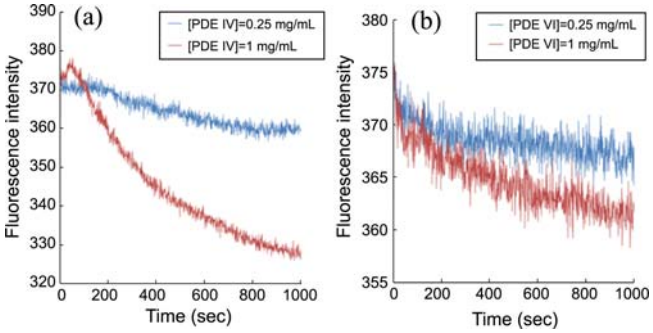



**Figure 1.** Real-time measurement of activities of **PDE** type IV (a) and **PDE** type VI (b) using cFMN (10 μM) and **PTZ-DPA** (50 μM) in pH 7.2 buffer solution (HEPES 10 mM, $MgCl_2$ 10 mM). [**PDE**] = 0.25 mg/mL, 1 mg/mL. Fluorescence intensity was recorded at 527 nm at 25 °C (excitation wavelength: 445 nm).

mL, 1 mg/mL). The fluorescence intensity of FMN at 527 nm was monitored in real-time by **PTZ-DPA** (Figure 1). The result indicates that the apparent reaction rate increases in proportion to the enzyme concentration. It turned out that PDE type IV is much more active than PDE type VI for hydrolysis of cFMN. This is the first example of PDEs hydrolyzing cFMN to FMN.

Next, we tested if **PTZ-DPA** can be used to screen the activity of a PDE inhibitor. We used 3-isobutyl-1-methyl xanthine (IBMX), a potent non-selective PDE inhibitor with an $IC_{50}$ value in the range 2-50 μM.[18] The real-time assay of PDE type IV and PDE type VI was performed in a mixture of cFMN (10 μM), **PTZ-DPA** (50 μM), and IBMX (100 μM) in a pH 7.2 buffer solution (10 mM HEPES, 1 mM $MgCl_2$). The stock solution of PDE was added to afford a final concentration of 1 mg/mL. PDE type IV also showed a more dramatic change than PDE type VI (Figure S1(a) and (b)). Figure S1(c) shows the PDE inhibition activity of IBMX at various concentrations (0, 10, 50, 100 μM). The decrease in the PDE enzymatic activity can be monitored by the change in fluorescence.

We were able to prove that PDE hydrolyzes cFMN to FMN by CE/ESI-MS (capillary electrophoresis/electro-spray ionization mass spectrometry). CE/ESI-MS is a rapid, high-resolution and high-efficiency method for the simultaneous detection of compounds, and we used it to follow the appearance of the peak of FMN as cFMN reacted with PDE. cFMN (100 μM) and PDE type IV (10 mg/mL solution containing 1 mM of $MgCl_2$) were incubated at 37 ºC for 1 h. Figure S2 shows that the intensity (*m/z* 437) of cFMN decreased and that (*m/z* 455) of FMN increased dramatically due to the PDE activity. The difference in *m/z* between the two peaks is 18 Da, which supports the hydrolysis of cFMN to FMN by PDE.

In conclusion, we presented real-time fluorescent detection of PDE activity by a chemosensor. The method introduced in this paper gives a convenient and real-time fluorometric assay of the hydrolysis of cFMN to FMN by PDEs. Moreover, the chemosensor can be successfully used for screening of a PDE inhibitor. Label-free fluorometric assaying of PDE and screening of PDE inhibitors has great potential in the identification of new biological pathways.

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