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Label-free fluorescent real-time monitoring of adenylyl cyclase

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ABSTRACT

In cellular signaling, adenylyl cyclase plays a key role in the hydrolysis of ATP to cyclic AMP and pyrophosphate. Using a synthetic fluorescent chemosensor (**PyDPA**) which binds strongly to the pyrophosphate group, we have developed a label-free fluorescent real-time detection system for adenylyl cyclase. This assay would be the first adenylyl cyclase assay based on chemosensing the production of pyrophosphate.

Cyclic adenosine 3′,5′-cyclic monophosphate (cyclic AMP or cAMP) is the most important second messenger, which is implicated in many biological processes including the metabolism of glycogen, sugar, and lipids.[1a] The regulation of cAMP formation is determined by adenylyl cyclase (EC 4.6.1.1, also known as adenylate cyclase or AC),[1b] which is a lyase enzyme that catalyzes the conversion of adenosine-5′-triphosphate (ATP) to cAMP and pyrophosphate (PPi).

Abnormal activities of adenylyl cyclase can cause fatal human diseases like heart disease[1c] or neurological disorders such as Alzheimer's disease.[1d] Therefore, assays for AC should be very accurate when related to disease diagnosis or drug development.

Several assay systems for AC have been developed. Many of them use radioactive ^{32}P-, ^{125}I- or fluorophore-labeled ATP as analogues of ATP to make radioactive or fluorescent cAMP, which can be detected after further separation through chromatography[2a] or capillary electrophoresis (CE).[2b] However, these methods are laborious, time-consuming, and difficult to apply to real-time monitoring of the enzyme. Furthermore, data from fluorophore-labeled ATP substrates tend not to be the same as that from natural ATP because the chemical structure of labeled ATP is different from that of natural ATP.

In other experiments, primary antibodies for cAMP have been used for radioimmunoassay.[2c,3] These methods are, however, very expensive because of the necessity of producing protein antibodies, and also hazardous due to the use of radioactive materials.

Almost all the known AC assay systems are based on the detection of cAMP produced from the enzyme reaction. Since AC produces not only cAMP but also PPi from ATP, our strategy of making a label-free assay system for AC is based on the detection of PPi released from the reaction. Recently, we reported that a synthetic receptor, a bis(Zn^{2+}-dipicolylamine) complex,[4,5] binds very strongly to PPi rather than to ATP in water.[4a,4b] Using this bis(Zn^{2+}-dipicolylamine) complex, we have developed fluorescent chemosensors which enable selective detection of PPi[4] or PPi-linked biomolecules.[5g,5h]

Among them, **PyDPA**, which was developed for ppGpp chemosensing,[5h] can also be utilized for AC assay. **PyDPA** binds more strongly to PPi ($K_a = 2.1 \times 10^8\ M^{-1}$) than to ATP ($K_a = 5.1 \times 10^6\ M^{-1}$). The binding affinity between cAMP and **PyDPA** was found to be negligible (not determined). Because the pyrene of **PyDPA** is environment-sensitive,[6] the adenine moiety of ATP could contribute to the increased hydrophobicity of the pyrene moiety of **PyDPA** in aqueous media (Fig. 1B), upon ATP's binding to **PyDPA**. In fact, fluorescence emission intensity of **PyDPA** (5.6 μM) showed eightfold enhancement upon the addition of ATP (Fig. 2). However, the addition of PPi to **PyDPA** caused only fourfold fluorescence emission increase because the bis(Zn^{2+}-dipicolylamine) moiety of **PyDPA** became rigidified[5d] upon the cooperative coordination of two sets of oxygen atoms of each P to the two Zn^{2+} ions,[4a,4b] just as in the case of the **PyDPA**–ATP complex. However, in comparison to ATP, PPi has no hydrophobic group that can help increase the hydrophobicity of the pyrene of **PyDPA** (Fig. 1A, Fig. 2). Since there is a considerable difference in the fluorescence emission intensity between **PyDPA**–ATP and **PyDPA**–PPi complexes, we expected that a label-free fluorescent real-time monitoring of

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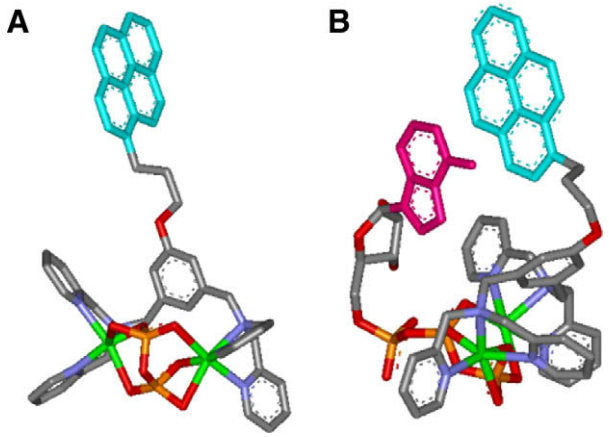


Figure 1. Computational energy-minimized modeling structures of (A) **PyDPA**–PPi complex and (B) **PyDPA**–ATP complex (SPARTAN'06).

AC would be possible (Scheme 1) using the different emission intensities between them.

Using an AC (type V) complex[3] which was freshly prepared from the striatum of a mouse brain, we tested whether **PyDPA** could be used for the real-time monitoring of AC. At each time point, the reaction mixture[7] was diluted (100×) in **PyDPA** solution (10 μM, 10 mM HEPES, pH 7.4) and the fluorescence of the diluted reaction mixture (total concentrations of ATP and PPi = 10 μM) in **PyDPA** solution (ex. 344 nm, em. 380 nm) was immediately recorded.

As the AC reaction proceeded, the amount of ATP decreased and that of PPi increased. As expected, the fluorescence intensity of **PyDPA** in the reaction mixture decreased over time. As the amount of AC (0.018 μg/μl, 0.045 μg/μl, 0.090 μg/μl) increased, the enzyme reaction rate gradually increased (Fig. 3A). Using the time-course fluorescence intensity (F) of **PyDPA**, we were able to calculate the ATP concentration ([ATP]) at each point in time from Eq. 1. Using the calculated [ATP] at each time, we analyzed each reaction rate as a pseudo-first-order reaction described by the Eq. 2:

$$[\mathrm{ATP}] = [\mathrm{ATP}]_0 \times [1 - (F_{\mathrm{ATP}} - F)/(F_{\mathrm{ATP}} - F_{\mathrm{PPi}})] \quad (1)$$

$$\ln[\mathrm{ATP}] = -k_{\mathrm{obs}}t + \ln[\mathrm{ATP}]_0 \quad (2)$$

(F_{ATP}: fluorescence (em. 380 nm, ex. 344 nm) of **PyDPA** upon the addition of 10 μM ATP, F_{ppi}: fluorescence of **PyDPA** upon the addition of 10 μM PPi, F: fluorescence of **PyDPA** upon the addition of a reaction mixture[7] at each reaction time, $[\mathrm{ATP}]_0$: initial concentration of ATP, 1 mM)

As shown in Figure 3B and C, the transformed plot from Eq. 2 shows good linearity with time and we could obtain pseudo-

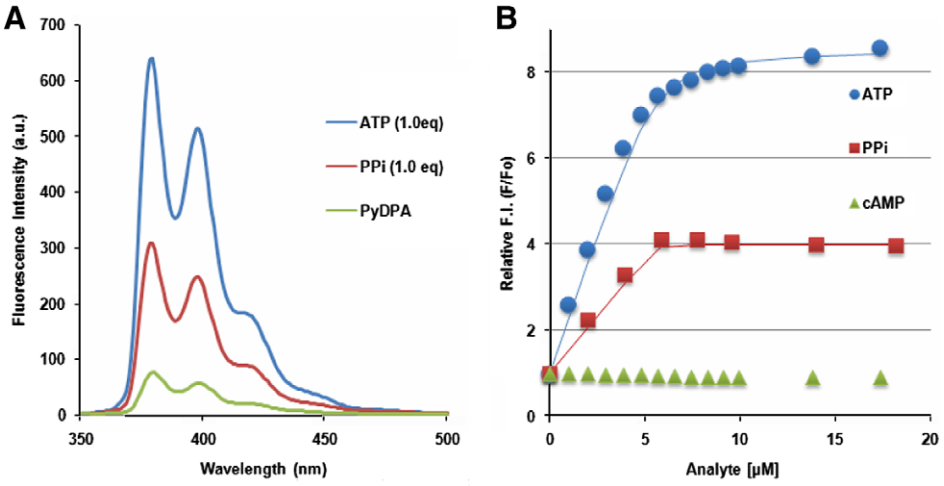
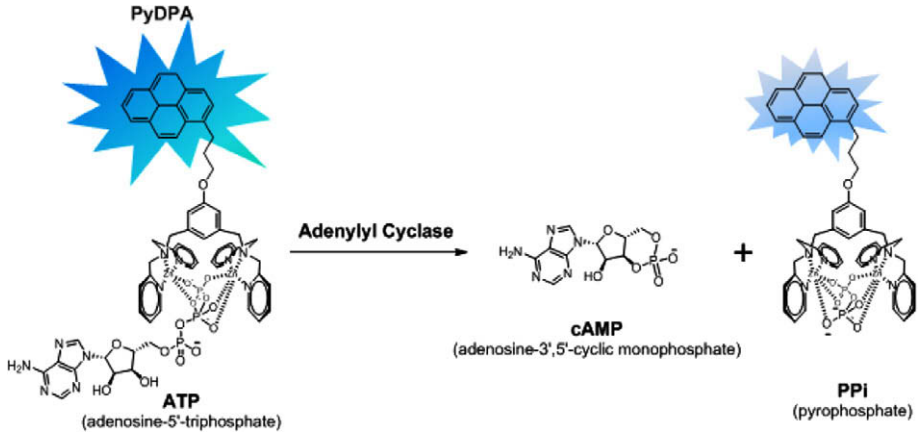


Figure 2. (A) Fluorescence emission of **PyDPA** (5.6 μM, 10 mM HEPES buffer, pH 7.4) with 1.0 equiv ATP or PPi (ex. 344 nm). (B) Titration of ATP, PPi, and cAMP with **PyDPA** (5.6 μM, 10 mM HEPES buffer, pH 7.4). Fluorescence intensities were recorded at 380 nm (ex. 344 nm).



Scheme 1. Fluorescent monitoring of AC activity with **PyDPA**.

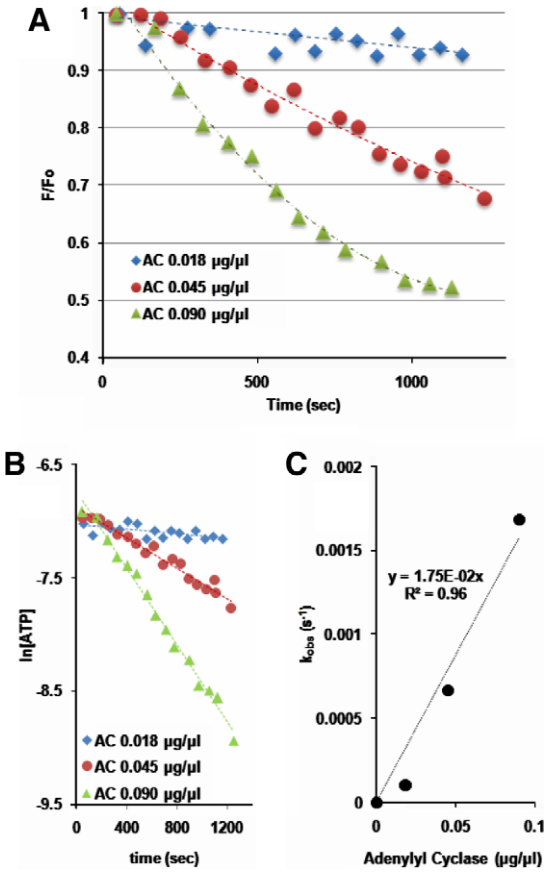


Figure 3. (A) Time–course measurement of fluorescence intensity of **PyDPA** (10 μM) according to variable amounts of AC (0.018 μg/μl, 0.045 μg/μl, 0.090 μg/μl). Fluorescence intensity was recorded at 380 nm (ex. 344 nm). (B) Pseudo-first-order rate kinetics of each enzyme reaction. (C) Observed rate constant (k_{obs}) values according to the amount of AC.

first-order rate constant values (k_{obs}) at each amount of AC (0.018 μg/μl, 0.045 μg/μl, 0.090 μg/μl), which are 1.04×10^{-4} s^{-1}, 6.68×10^{-4} s^{-1} and 1.68×10^{-3} s^{-1}, respectively. The data were comparable with other reported k_{obs} values (5.83×10^{-4} s^{-1}) of AC reactions obtained by the conventional detection method.[8]

The conversion of another purine nucleotide, guanosine-5′-triphosphate (GTP), to cyclic guanosine 3′,5′-cyclic monophosphate (cGMP) and PPi was also tested using the same enzyme, the AC complex. It turned out that the AC (type V) complex hydrolyzed GTP more slowly than ATP (Fig. 4). Further, we observed that AC could not perform efficiently in the absence of creatine phosphokinase (CPK, 8.0 U/ml), a component of the ATP regenerating-system which stimulates the activity of adenylyl cyclase (Fig. 4).[9]

In conclusion, we have developed a label-free fluorescent real-time detection system for AC assay using the distinct difference in fluorescence emission intensities between **PyDPA**–ATP and **PyDPA**–PPi complexes. This is the first adenylyl cyclase assay system which is based on fluorescent chemosensing of the production of PPi.

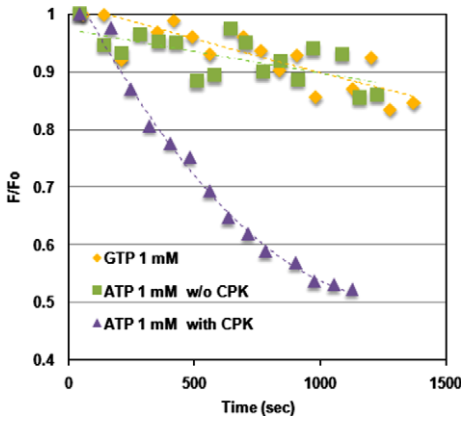


Figure 4. Real-time measurement of AC (9.0 μg) with GTP (1 mM), ATP (1 mM) in the presence or absence of CPK (creatine phosphokinase). Fluorescence intensity was recorded at 380 nm (ex. 344 nm).

Acknowledgments

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7. 80 mM Tris–HCl (pH 7.4), 1 mM ATP, 10 μM forskolin, 1 mM creatine phosphate, 0.8 U creatine phosphokinase, 1 mM $MgSO_4$, 1 mM EGTA, 30 mM NaCl, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5 mM dithiothreitol (DTT), 1.8, 4.5 or 9.0 μg AC (V) complex. Total reaction mixture volume = 100 μl.
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