Cation Effect on Fluorescent Sensing of Pyrophosphate by a Bis(Zn–DPA) Probe

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Received May 8, 2018, Accepted May 18, 2018, Published online June 19, 2018

Keywords: Pyrophosphate, Fluorescent probes, Buffer, Cation, Binding mode

Pyrophosphate ($P_2O_7^{4-}$, PPi) is a key metabolic byproduct produced by some enzymes, such as nucleic acid polymerases, adenylyl cyclase, and aminoacyl–tRNA synthetases. These enzymes are of high interest because they are closely related to not only cellular proliferation and growth but also several diseases including cancers.[1,2] Moreover, an abnormal concentration of PPi in body fluid often causes pseudogout, osteoarthritis, and urolithiasis.[3,4] Therefore, there is growing interest in monitoring the activities of these enzymes. The importance of PPi in bioanalytical and clinical aspects urged chemists to develop selective and sensitive detection methods for PPi.[5–7] In order to analyze the activities of these enzymes, it is highly desirable to measure the concentration of PPi precisely under complex physiological conditions involving various ions and a large excess of competing biological phosphates such as adenosine 5′-triphosphate (ATP).

However, the majority of PPi probes, including bis(Zn–DPA) (DPA = dipicolylamine) complexes such as **1**·2Zn[8] (Scheme 1), was tested in a media of low ionic strength, while many enzymatic reactions require high ionic strength.[8–10] Therefore, a study on the effects of media toward selectivity and sensitivity is necessary to develop a highly selective and sensitive probe for PPi that is suitable for its real-time monitoring. In that regard, it is worth noting that Jolliffe's macrocyclic host molecules (*e.g.,* **2**·Zn_2,[11] Scheme 1) showed higher selectivity for PPi in the biologically relevant Krebs buffer system than in low ionic strength buffers.[11–13] Nevertheless, the underlying principles for why they show high selectivity are still unclear. Therefore, we investigated how the buffer composition affected molecular recognition of PPi and ATP.

Normal Krebs buffer (pH 7.4) contains a number of ions such as sodium (Na^+, 137 mM), potassium (K^+, 5 mM), magnesium (Mg^{2+}, 1.2 mM), calcium (Ca^{2+}, 2.8 mM), chloride (Cl^-, 150 mM), sulfate (SO_4^{2-}, 1 mM), and phosphate (PO_4^{3-}, 1 mM) as well as non-ionic glucose (10 mM). Because aforementioned glucose and anions would have insignificant effects on the molecular recognition of PPi, it is without doubt that cations (especially Mg^{2+} and Ca^{2+}) play critical roles in modulating association constants.

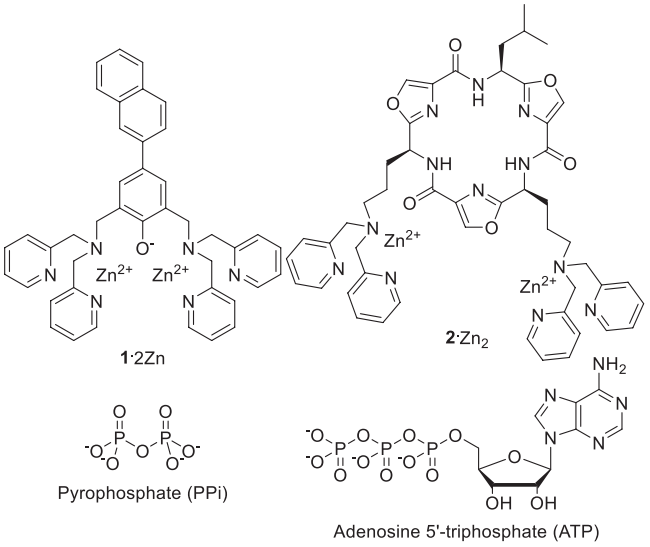
Therefore, we conducted a series of titration experiments in several media containing individual cations. Since probe **1**·2Zn showed considerable signal enhancement in the presence of either PPi or ATP as depicted in Figure 1**,** **1**·2Zn is a suitable probe for determining association constants (Figures S1–S3, Supporting information). Table 1 summarized the results obtained by fitting the fluorescence intensity at 470 nm (I_{470}) against the analyte concentrations (Figure 2, Figures S4–S6, see the Appendix S1 for detailed methods for fitting). It is noticeable that log $K_{a,PPi}$ − log $K_{a,ATP}$ (difference of two individual log K_a's) and log K_{PPi}/K_{ATP} (obtained by competition experiment) did not fully agree with each other (Table S1). However, we believe that the latter one is more significant because it directly reflects the ability to detect PPi in the presence of large amounts of ATP.

Addition of 100 mM NaCl slightly reduced the association constant between **1**·2Zn and analytes (Table 1), which supports the fact that intermolecular forces predominantly rely on ion-ion interactions.[14] However, there is only a little change in association constants, indicating that sodium and chloride ions would have negligible effects on binding of **1**·2Zn to PPi and ATP.

Surprisingly, alkaline earth metal ions, even at 5 mM concentration, efficiently suppressed the association between **1**·2Zn and analytes without inducing a substantial increase in the association constant ratio (log $K_{a,PPi}/K_{a,ATP}$). This phenomenon is clearly different from the binding behavior of Jollife's peptoid hosts, since many of them showed enhanced selectivity in Krebs buffer system.[7] Therefore, we thought that the selectivity difference between ours and Jolliffe's would be attributed to not only the action of divalent cations but also the molecular structure of host molecules.[7]

In order to understand how the alkaline earth metal ions modulate the association constants between **1**·2Zn and analytes, we calculated the association constants by assuming that the ions and **1**·2Zn competitively bind to analytes, and the results are summarized in Table S2. The new association constants obtained from the solution containing Mg^{2+} ions agreed with previous ones within the error range. However, the association constants in the presence of Ca^{2+}

ISSN (Print) 0253-2964 | (Online) 1229-5949



Scheme 1. Molecular structures of **1**·2Zn, **2**·Zn_2, pyrophosphate (PPi), and adenosine 5′-triphosphate (ATP).

ions were either one order of magnitude smaller (7.2 ± 0.1 for PPi) or one-third (6.1 ± 0.2 for ATP) compared to those determined previously (8.4 ± 0.3 and 6.6 ± 0.2, respectively). Therefore, it is highly likely that Ca^{2+} ions participate in complexation with the diphosphate unit of PPi and ATP in a different way when compared to the binding mode of Mg^{2+} ions (*vide infra*).

It has been known that many crystal structures of biomacromolecules exclusively carry a unique six-membered ring structure composed of a Mg^{2+} and a PPi ion (MgPPi),[15–17] which is structurally analogous to the complex between PPi and **1**·2Zn.[18] However, a Ca^{2+} ion sometimes forms a 4-membered ring structure with PPi in a side–on manner at the phosphate unit.[19] This is perhaps not a coincidence

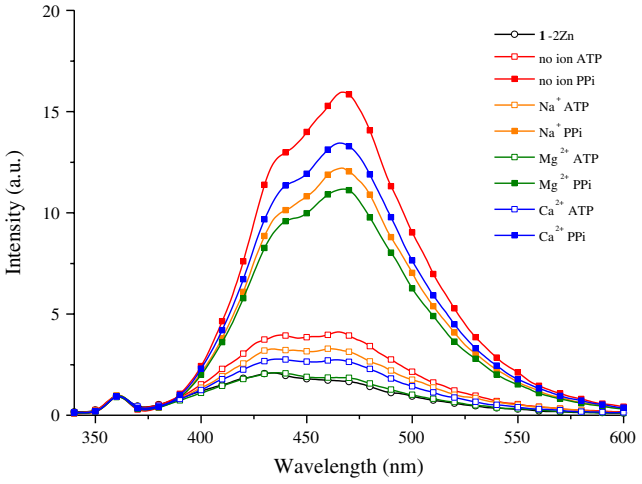


Figure 1. Fluorescence responses of **1**·2Zn (8 μM) to analytes (5 equiv.) in various media: No ion, 50 mM HEPES; Na^+, 50 mM HEPES, 100 mM NaCl; Mg^{2+}, 50 mM HEPES, 5 mM $MgCl_2$; Ca^{2+}, 50 mM HEPES, 5 mM $CaCl_2$. All HEPES buffers were set at a pH of 7.4.

Table 1. Apparent association constants (log K_a) between **1**·2Zn and analytes in 50 mM HEPES buffer (pH 7.4) at various ionic concentrations.[a]

Added ion	PPi[b]	ATP[b]	log $K_{a,PPi}/K_{a,ATP}$[c]
None	8.4 ± 0.3	6.6 ± 0.2	1.3 ± 0.05
100 mM NaCl	8.1 ± 0.2	6.2 ± 0.2	1.5 ± 0.05
5 mM $MgCl_2$	6.0 ± 0.05	4.9 ± 0.5	0.8 ± 0.05
5 mM $CaCl_2$	6.3 ± 0.1	5.2 ± 0.2	1.6 ± 0.05

[a] The association constant was averaged from at least two individual titration experiments.
[b] Determined by fitting the fluorescence intensity at 470 nm according to 1:1 binding.
[c] Determined by fitting the fluorescence intensity according to competitive binding of two guests to a host.

considering the fact that the ionic radii of Mg^{2+} (86 pm) is similar to that of Zn^{2+} (88 pm) whereas Ca^{2+} (114 pm) is about 30% bigger.[20] Therefore, one can expect that binding of **1**·2Zn and Mg^{2+} ion to PPi only takes place at the same location with the same geometry (between two phosphate groups), while Ca^{2+} can bind to PPi at a different position (at a phosphate group). Consequently, Mg^{2+} ions necessarily compete with **1**·2Zn for binding PPi, whereas Ca^{2+} ions can inhibit the binding between **1**·2Zn and PPi both competitively and uncompetitively. That's why the association constant between **1**·2Zn and PPi decreased in the presence of Ca^{2+} ions (Table S2).

A recent study claimed that a three-point contact through three oxygen atoms of each phosphate (P_α, P_β, P_γ) is the most favorable conformation of Mg^{2+}–bound ATP (MgATP).[21] Therefore, an MgATP complex in the most stable conformation must release two consecutive points of contact before binding to a host molecule, where Mg^{2+} ion still can be attached to ATP, preferentially at the P_α oxygen. Therefore, one can rationally imagine that the proximal hydroxyl group of the ribosyl moiety and water molecules can fill the empty coordinating sites of the Mg^{2+} ion, resulting in a large hydration sphere in which way ATP becomes more sterically hindered. An energy-minimized structure of the **1**·2Zn-ATP complex clearly reveals that the adenosine monophosphate (AMP) unit of ATP is located far away from **1**·2Zn and thus can be solvated freely (Figure S7). However, the AMP unit of the **2**·Zn_2-ATP complex experiences severe steric interference with **2**·Zn_2 (Figure S8). In other words, it is more likely that **2**·Zn_2 encounters steric interactions with the AMP unit. Combining two arguments, we can expect that the sterically demanding MgATP must lose affinity to a greater extent for **2**·Zn_2.

In conclusion, we conducted a series of titration experiments to analyze the effects of metal ions in Krebs buffer on the selectivity and sensitivity of PPi probes. The selectivity is certainly attributed to not only the action of divalent cations but also the molecular structure of the host molecules. We confirmed that divalent cations competitively bind PPi with host molecules, while Ca^{2+} participates in complexation with

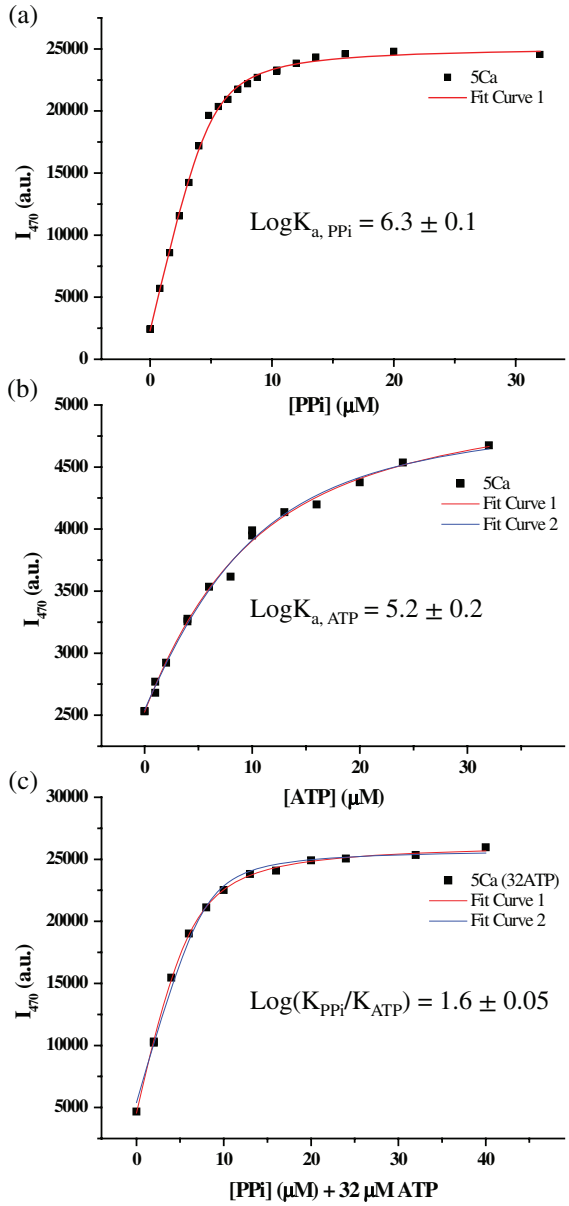


Figure 2. Representative fitting results of the fluorescence intensity at 470 nm (I_{470}) of **1**·2Zn (8 μM) against various concentrations of (a) PPi, (b) ATP, and (c) PPi (in the presence of 32 μM ATP) in 5 mM $CaCl_2$, 50 mM HEPES buffer (pH 7.4).

host molecules in an additional uncompetitive manner. Moreover, we rationalized the reason why Jolliffe's macrocyclic host molecules have greater selectivity for PPi in Krebs buffer. A study to improve PPi selectivity by the synergy effect of divalent cations and steric effect is under way using a macrocyclic derivative of **1**·2Zn.

Experimental

General information. Fluorescence emission spectra were obtained on a SCINCO FluoroMate FS-2 fluorescence spectrometer and the slit width was 10 nm for excitation and 5 nm for emission. The **1**·2Zn solution for all the photophysical experiments was prepared from 10 mM stock solution in DMSO, diluted with appropriate buffer solution. Obtained data were fitted with OriginPro 8 program. The molecular structure computation was performed by Spartan '08 program.

Acknowledgments. This work was supported by the National Research Foundation of Korea (NRF) grant funded by MSIP (No. 2015M3A6A4076701).

Supporting Information. Additional spectral data, fitting results and association constants are included.

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