Visual Detection of Di- and Tri-phosphates in Aqueous Solution of Neutral pH

Min Su Han and Dong H. Kim *

Center for Integrated Molecular Systems and Department of Chemistry, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31 Hyojadong, Pohang 790-784, Korea
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The chemosenssor that consists of [Zn_2(1,3-*bis*[*bis*(2-pyridylmethyl)aminomethyl]benzene)]$^{4+}$ (receptor) and pyrocatechol violet (signaling unit) detects with naked eyes di- and tri-phosphates conjugated to nucleosides or in free forms. The blue color of the aqueous solution (pH 7.0) of the sensor turns to yellow upon exposing to the analytes.

Key Words : Chemosensor, Diphosphate, Triphosphate, ATP, ADP

Introduction

Development of chemosensors is a burgeoning field of chemistry as an outgrowth of molecular recognition which has made an impressive progress in last several decades.[1] Most of chemosensors are assembled by appending a signaling unit to a receptor molecule. The receptor recognizes and binds a target molecule (analyte) and the signaling unit acts as a signal transducer by converting the recognition event into a signal. Of numerous types of non-covalent bondings that are involved in the interactions between the chemosensors and analytes, coordinative interaction is of special interest due to its high binding affinity compared with the other binding interactions.[2] Thus, the metal-ligand interaction has been utilized for designing chemosensors that detect anions in aqueous media.[2]

We have been interested in developing chemosensors for anions that play important roles in the biological systems.[3] In this paper, we wish to report a chemosensor that can detect visually as well as spectrophotometrically diphosphate or triphosphate in aqueous solution of neutral pH.

Results and Discussion

In designing the present probe we have applied the displacement strategy developed by Anslyn.[4] In the strategy, a chromogenic signaling unit (indicator) is bound non-covalently to a receptor that can recognize and form a complex with an analyte of interest. The analyte is then allowed to compete for the receptor with the signaling unit. The successful displacement of the receptor-bound signaling unit by the analyte results in displaying a spectral modulation that can be communicated spectroscopically (Figure 1). Thus, the receptor should be chosen in such a way that it can bind both the signaling unit and the analyte, but with stronger interactions with the analyte, and the spectrum of the indicator that is bound to the receptor should be different significantly from that of the indicator in a free form. In designing the present probe, [Zn_2(1,3-*bis*[*bis*(2-pyridyl-methyl)aminomethyl]benzene)]$^{4+}$ (**1**) was chosen to serve as the receptor and pyrocatechol violet (**2**) as a signaling unit. The two zinc ions in the receptor are thought to interact with

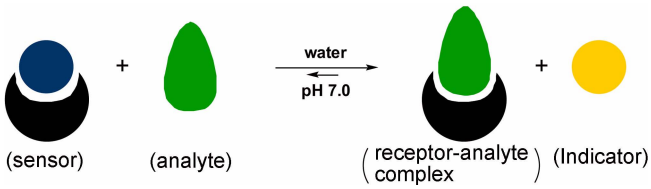
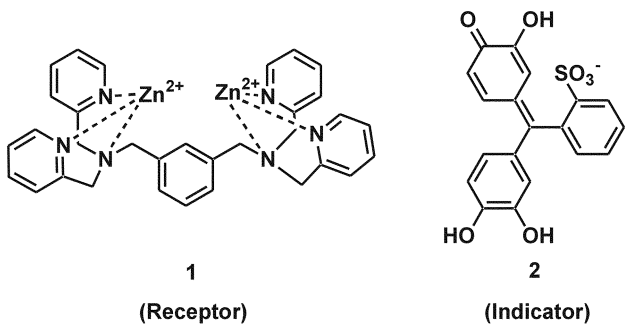


Figure 1. A schematic illustration for chemosensors that can be assembled by the displacement protocol.



1 (Receptor) **2** (Indicator)

the two phenolic hydroxyls in the signaling unit but more strongly with a di- or triphosphate moiety. The yellow color of pyrocatechol violet solution of neutral pH is shown to change to blue upon binding it to a metal ion.[5] Therefore, it is expected that the displacement of the receptor-bound signaling unit by the analytes would be communicated visually from the color change of blue to yellow. The displacement may also be detected spectrophotometrically from a change in the Uv-visible absorbance.

The probe was obtained by simply mixing (1,3-*bis*[*bis*(2-pyridylmethyl)aminomethyl]benzene)] (BBPAB),[6] zinc perchlorate, and **2** in an 1 : 2 : 1 molar ratio in the aqueous solution of HEPES buffer pH 7.0. Figure 2 shows the UV-visible spectra obtained when the solution of **1** was titrated into the aqueous buffer (pH 7.0) of **2** (25 μM). As the

*To whom correspondence should be addressed. Fax: +82-54-279-8142 or +82-54-279-5877, e-mail: dhkim@postech.ac.kr

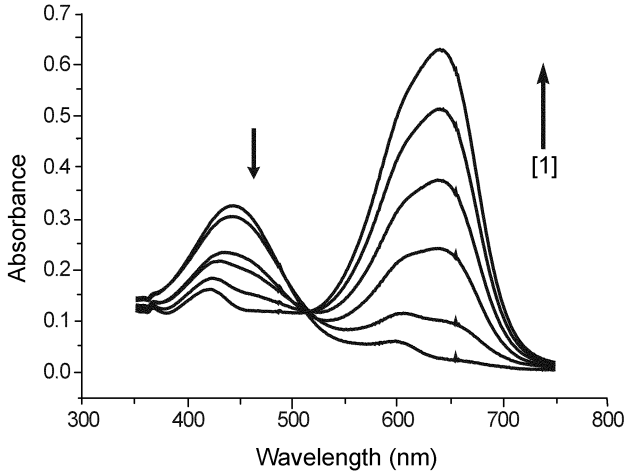


Figure 2. Uv-visible spectra obtained upon addition of receptor (**1**) solutions (final concentrations: 0, 5, 10, 15, 20, and 25 μM) to the pH 7.0 aqueous buffer (HEPES, 10 mM) containing pyrocatechol violet (**2**) (25 μM).

concentration of **1** increases, the absorbance at 444 nm that is due to **2** is diminished and there appears a new absorption at 640 nm, indicating the proposition that the signaling unit (**2**) forms a complex with the receptor, most likely its phenolic hydroxyls coordinating to the zinc ions in the receptor.

The association constant for the binding of **2** to **1** was determined to be (5.74 ± 1.1) × 10^6 M^{-1} by isothermal titration calorimetry[7] (ITC) (Figure 3). From the ITC experiment the thermodynamic parameters for the binding of **2** to **1** in the aqueous solution of pH 7.0 (HEPES 10 mM) at 30 °C were also obtained as the following: ΔH = –(5.08 ± 0.04) kcal mol^{-1} and ΔS = +14.2 eu, indicating that the binding is favored enthalpically as well as entropically. The stoichiometric coefficient value (n) was determined to be 1.23, suggesting the 1 : 1 binding mode in the complex formation, which was confirmed by the Job plot[8] (Figure 4).

Detailed study for the binding of the receptor to triphosphate was made with adenosine triphosphate (ATP), a representative nucleoside triphosphate. Changes in the UV-visible absorption spectra upon additions of ATP to the solution of the sensor are shown in Figure 5: upon the addition of ATP, the absorbance at 640 nm decreases progressively, while the peak at 444 nm increases, which is expectedly exact reverse of the phenomenon observed when **1** was added to the solution of **2**, and indicates that the ATP displaces the receptor-bound indicator. In determining the association constant for the binding of ATP to the receptor, we employed the method developed by Kuzmič *et al*. and used for the determination of the binding constant of cyclophilin to cyclosporin A using a fluorescent derivative D-Lys(Dns)-cyclosporin A.[9] The binding constant of (6.46 ± 0.22) × 10^8 M^{-1} was thus obtained. As anticipated, the binding affinity of ATP towards **1** is greater than that of **2** towards **1** by 113-fold. The pH dependence of absorbance change of the sensor in the absence and presence of ATP was studied to find that the maximum change in the UV-visible

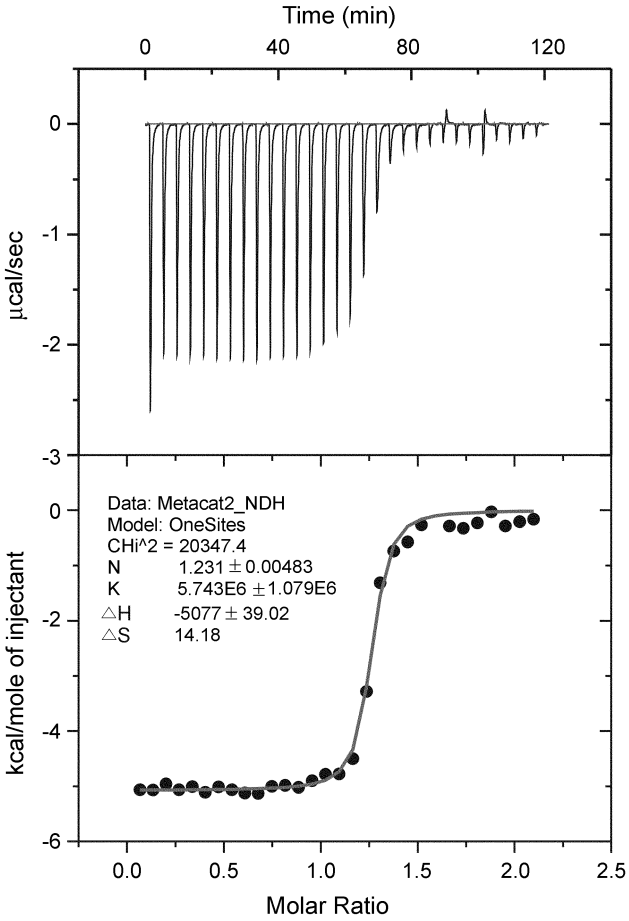


Figure 3. The ITC plot for the titration of receptor (**1**) solution with indicator (**2**) in aqueous buffer (HEPES) pH 7.0 at 30 °C.

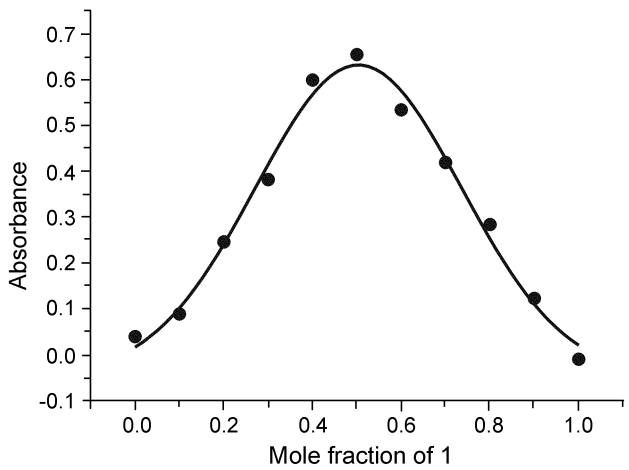


Figure 4. Job plot for binding of pyrocatechol violet (**2**) with the receptor (**1**) in a pH 7.0 (HEPES 10 mM) buffer solution. Aqueous solutions of the receptor (50 μM) and pyrocatechol violet (50 μM) were mixed in varying ratios and the changes of absorbance at 640 nm were measured.

absorption is observable at pH 6.5-7.0 (Figure 6).

The sensor shows a negative response to adenosine monophosphate (AMP). Although the extent of the UV-visible absorbance lowering caused by adenosine diphosphate

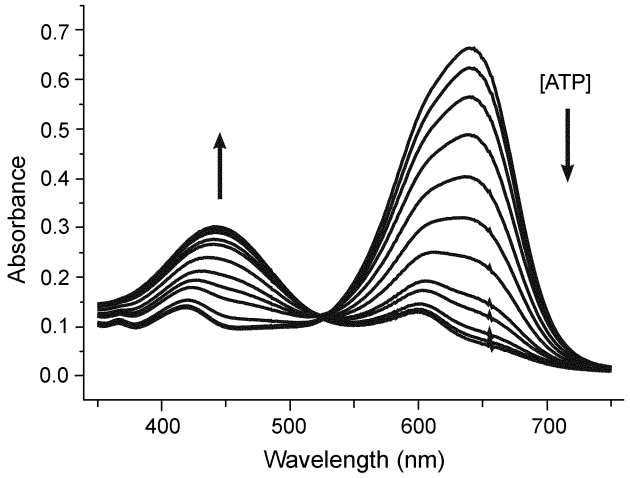


Figure 5. Uv-visible spectra obtained by additions of ATP solutions (final concentrations: 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33 μM) to the pH 7.0 aqueous buffer (HEPES, 10 mM) containing the sensing ensemble, $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM).

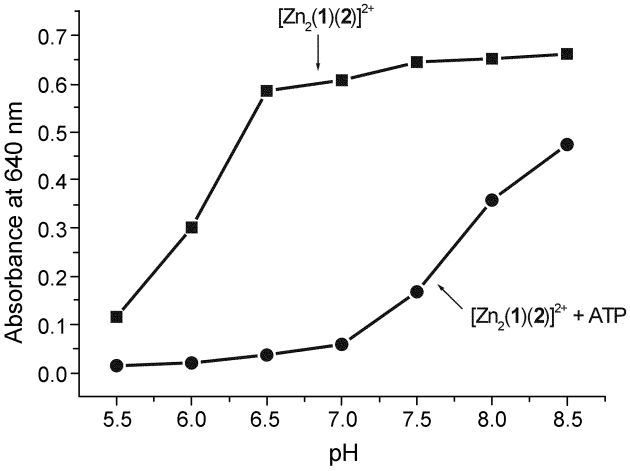


Figure 6. Uv-visible absorbances of the aqueous solution of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) and $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ with ATP (25 μM) at various pHs. Compositions of the buffer solutions are as the following: pH 5.5 (MES 10 mM), pH 6.0 (MES 10 mM), pH 6.5 (MES 10 mM), pH 7.0 (HEPES 10 mM), pH 7.5 (HEPES, 10 mM), pH 8.0 (HEPES 10 mM), and pH 8.5 (CHES 10 mM).

(ADP) is considerably less compared with that caused by ATP, it is not sufficient to differentiate ATP from ADP in a qualitative detection (Figure 7). In an effort to understand the difference shown by the two phosphates in lowering of the UV-visible absorption, we have determined the association constant for the binding of ADP to the receptor and compared it with that for the receptor-ATP complex. The association constant of $(1.22 \pm 0.04) \times 10^7$ M^{-1} for the binding of ADP to the receptor was obtained by the non-linear regression analysis of the titration data obtained by titrating ADP solution to the aqueous buffer (pH 7.0) containing $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$. Thus, the binding affinity of ATP to the receptor is greater than ADP by 53-fold. The result may be rationalized with the proposition that the negatively charged oxygen ions in the phosphates play important roles,

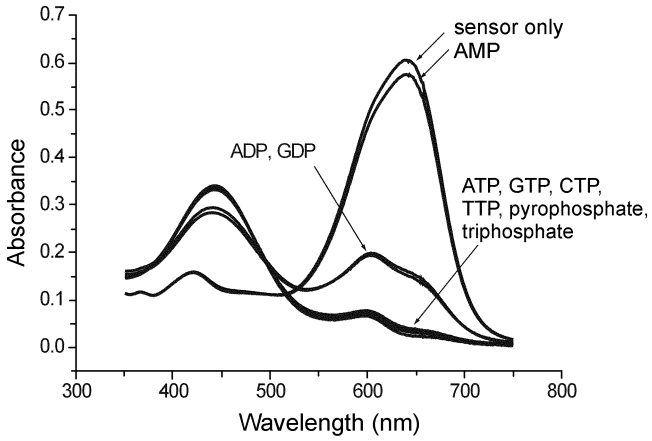


Figure 7. Uv-visible spectra of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) in the presence of monophosphate, diphosphate, and triphosphate (25 μM) in pH 7.0 aqueous buffer (HEPES) solution.

possibly interacting with the two Zn^{2+} ions in the receptor to form coordinative bonds between the oxygen atoms and the zinc ions. It has been well established that Zn^{2+} can accommodate up to 6 coordination numbers.[10] Thus, ATP that bears four negatively charged oxygen atoms binds the receptor more strongly than ADP which carries only three negatively charged oxygen atoms. It is noteworthy that the lowering extent of UV-visible absorbance caused by unconjugated diphosphate, *i.e.*, pyrophosphate is greater than those by ADP and GDP, reaching the level essentially the same as those caused by the triphosphate of nucleosides, *i.e.*, ATP, GTP, CTP, and TTP (Figure 7). The strong binding interaction of the pyrophosphate with the receptor may be envisioned on the ground that pyrophosphate carries four negatively charged oxygen atoms. Commonly found organic and inorganic anions such as carbonate, acetate, phosphate, sulfate, nitrate, and chloride show negative responses (Figure 8). The presence of a large amount of other ions such as chloride, sulfate and sodium ions hardly affects the selectivity shown by the present sensor (Figure 9).

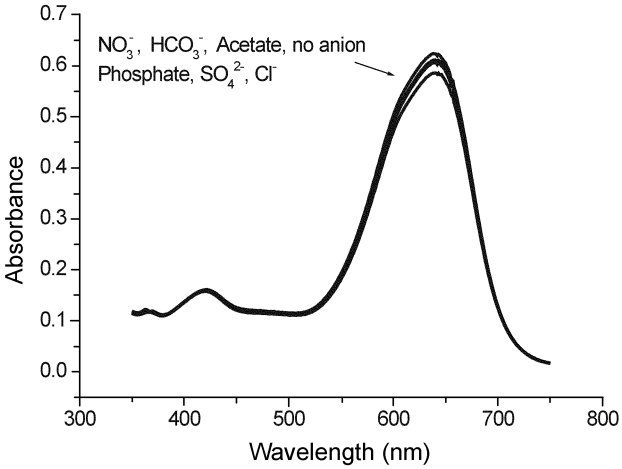


Figure 8. Uv-visible spectra of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) in the presence of a variety of anions (25 μM) in pH 7.0 aqueous buffer (HEPES) solution.

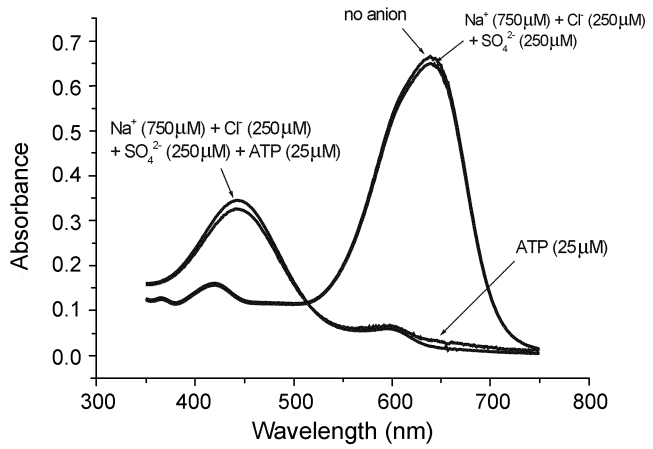


Figure 9. Uv-visible spectra of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) in the presence of a large excess of anions and a cation in pH 7.0 aqueous buffer (HEPES) solution.

Figure 10. Color shown by $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) in the absence and presence of anions (25 μM). From left to right: no anion, ATP, ADP, AMP, HPO_4^{2-}, SO_4^{2-}, CH_3CO^{2-}, HCO_3^-, Cl^-, and NO_3^-.

Sensors that can detect analytes by naked eye without resorting to a spectrometric instrument is of particular interest because of the convenience in its usage. The use of the present ensemble for such purpose has been demonstrated in Figure 10: the color change from blue to yellow was observed only when ADP or ATP is added to the aqueous solution of the sensor. Other anions failed to cause the color change.

In summary, we have designed a probe that can detect nucleoside triphosphate and diphophate as well as free triphosphate and diphosphate (pyrophosphate) in aqueous media of neutral pH. All other commonly encountered anions show negative responses. The detection can be made either from the decrease of the absorbance at 640 nm or visually from the development of yellow color. The sensing ensemble can be prepared easily by simply mixing BBPAB, zinc perchlorate, and pyrocatechol violet in an 1 : 2 : 1 molar ratio in water of neutral pH.

Experimental Section

All reagents used in this work were purchased from Sigma-Aldrich Chemical Co. and were used without further purification. (1,3-*bis*[*bis*(2-Pyridylmethyl)aminomethyl]-benzene)] (BBPAB) was prepared as described in the literature.[6] Buffer solutions were prepared with deionized water. UV-visible spectra were obtained with a Hewlett-Packard 8453 diode array spectrophotometer.

Determination of the K_{ass} Value for the Binding of ATP to 1. The competitive complexation equilibrium between receptor (R), indicator (I), and analyte (A) in solution can be expressed by Eq. (1):

$$R{\cdot}I + A \rightleftharpoons R{\cdot}A + I \tag{1}$$

The mass balance and the dissociation constants of the competitive complexation equilibrium are defined by Eqs. (2)-(6):

$$R + I \rightleftharpoons R{\cdot}I \quad K_{d1} = \frac{[R][I]}{[R \cdot I]} \tag{2}$$

$$R + A \rightleftharpoons R{\cdot}A \quad K_{d2} = \frac{[R][A]}{[R \cdot A]} \tag{3}$$

$$[A]_T = [A] + [R{\cdot}A] \tag{4}$$

$$[R]_T = [R] + [R{\cdot}A] + [R{\cdot}I\,] \tag{5}$$

$$[I]_T = [I] + [R{\cdot}I] \tag{6}$$

The free receptor concentration [R] can be obtained from the mass balance and the dissociation constants as expressed by Eq. (7):

$$[R] = \frac{[R]_T}{1 + ([I]_T/K_{d1} + [R]) + ([A]_T/K_{d2} + [R])} \tag{7}$$

In the competitive equilibrium above, R·I exhibits an intense UV absorption at 640 nm. If $[I]_T$ is the same in all solutions used in the experiment, the absorbance (A) at any finite concentration of R can be determined by the molar fraction of I in the bound state according to Eq. (8):

$$[A] = A_I + (A_{RI} - A_I)\frac{[R \cdot I]}{[R \cdot I] + [I]} \tag{8}$$

in which A_{RI} denotes the absorbance at competitive saturation and A_I represents absorbance of I. Since $K_{d1} = [R][I]/[R{\cdot}I]$, Eq. (8) can be converted into Eq. (9) and rearrangement of Eq. (9) gives Eq. (10):

$$A = A_I + (A_{RI} - A_I)\frac{[R]}{[R] + K_{d1}} \tag{9}$$

$$[R] = K_{d1}\frac{A - A_I}{A_{RI} - A} \tag{10}$$

Substitution for [R] from Eq. (10) into Eq. (7) and rearrangement gives Eq. (11), with

$$A = A_I + (A_{RI} - A_I)\left[[R]_T/[R]_T + \frac{A_{RI} - A}{A_{RI} - A_I}[I]_T + \frac{1}{K_{d2}/K_{d1} + (A - A_I)/(A_{RI} - A)}[A]_T + K_{d1}\right] \tag{11}$$

which the dissociation constant of $(1.55 \pm 0.02) \times 10^{-9}$ M for **1**·ATP was obtained by the non-linear regression analysis using the software Daynafit.[11] Reciprocal of the dissociation constant, $(6.46 \pm 0.22) \times 10^8$ M^{-1} denotes the binding

constant for the binding of ATP to **1**. For the analysis of Eq. (11), K_{d1} (the dissociation constant of **2**·**1**) and the titration data (concentration of ATP and absorbance at 640 nm) are required. The dissociation constant of **2**·**1** was determined using an isothermal titration calorimeter (ITC) to be 1.72×10^{-7} M. The titration data was obtained as follows: A 3.0 mL solution containing **1** (25 μM) and **2** (25 μM) in pH 7.0 buffer (HEPES, 10 mM) was prepared, and its UV absorbance at 640 nm was measured. Aliquots of ATP solution in pH 7.0 buffer (HEPES, 10 mM) were titrated into the solution to give final concentrations of 3.0-33.0 μM and the absorbance at 640 nm were recorded. In a similar fashion, the K_{ass} for the binding of ADP to **1** was determined.

Determination of the Stoichiomtry for Binding of 2 to 1 to Form the Sensing Ensemble. Aqueous solutions of **1** (50 μM) and **2** (50 μM) in pH 7.0 buffer (HEPES, 10 mM) were mixed in varying ratios and the changes of absorbance at 640 nm were measured. The stoichiometry was obtained from the titration plot (Job's plot) of the absorbance at 640 nm (Figure 4).

pH Dependence of the Sensing. Varying pH (pH 5.5-8.5) buffer solutions of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) and solutions of $[Zn_2(\mathbf{1})(\mathbf{2})]^{2+}$ (25 μM) with ATP (25 μM) were prepared, and their UV-visible absorbances at 640 nm were measured (Figure 6) and plotted the abosrbances against pH to obtain Figure 6. The compositions for the buffer solutions are as follows: pH 5.5 (MES 10 mM), pH 6.0 (MES 10 mM), pH 6.5 (MES 10 mM), pH 7.0 (HEPES 10 mM), pH 7.5 (HEPES, 10 mM), pH 8.0 (HEPES 10 mM), and pH 8.5 (CHES 10 mM).

Determination of Thermodynamic Parameters for the Binding of 2 to 1 with an Isothermal Titration Calorimeter. An aqueous solution (1.5 mL, pH 7.0, HEPES 10 mM) of **1** (0.1 mM) was added to the sample cell of an isothermal titraton calorimeter and aqueous buffer solution (1.7 mL, pH 7.0, HEPES 10 mM) was added to the reference cell. Into the solution in the sample cell was injected 7 μL portions of aqueous solution of **2** (2.0 mM) in 30 times with 200 sec interval. Each mixture was continuously stirred at 300 rpm and kept at an operating temperature of 30 °C. After each addition of the aqueous solution of **2**, the heat (μcal s^{-1}) released in the sample cell was measured with respect to a reference cell. The data were analyzed using the software Origin to give Figure 3.

References

1. (a) Beer, P. D.; Schmitt, P. *Curr. Biol. Chem. Biol*. **1997**, *1*, 475. (b) Beer, P. D.; Gale, P. A. *Angew. Chem. Int. Ed*. **2001**, *40*, 486. (c) Masrtinez-Mánez, R.; Sancenón, F. *Chem. Rev.* **2003**, *103*, 4419.
2. (a) Aoki, S.; Kimura, E. *Rev. Mol. Biotech.* **2002**, *90*, 129. (b) Fabbrizzi, L.; Leone, A.; Taglietti, A. *Angew. Chem. Int. Ed.* **2001**, *40*, 3066. (c) Fabbrizzi, L.; Marcotte, N.; Stomeo, F.; Taglietti, A. *Angew. Chem. Int. Ed*. **2002**, *41*, 3811. (d) Amendola, V.; Fabbrizzi, L.; Mangano, C.; Pallavicini, P.; Poggi, A.; Taglietti, A. *Coord. Chem. Rev.* **2001**, *219-221*, 821. (e) Cha, K. W.; Huang, H. Z.; Choi, H. C. *Bull. Korean Chem. Soc.* **2002**, *23*, 1456.
3. (a) Han, M. S.; Kim, D. H. *Angew. Chem. Int. Ed.* **2002**, *41*, 3809. (b) Han, M. S.; Kim, D. H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1079. (c) Kim, D. H.; Han, M. S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2543.
4. (a) Wiskur, S. L.; Ait-Haddou, H.; Lavigne, J. J.; Anslyn, E. V. *Acc. Chem. Res*. **2001**, *34*, 963. (b) Ait-Haddou, H.; Wiskur, S. L.; Lynch, V. M.; Anslyn, E. V. *J. Am. Chem. Soc*. **2001**, *123*, 11296. (c) Wiskur, S. L.; Anslyn, E. V. *J. Am. Chem. Soc*. **2001**, *123*, 10109. (d) Metzger, A.; Anslyn, E. V. *Angew. Chem. Int. Ed*. **1998**, *37*, 649. (e) Niikura, K.; Metzger, A.; Anslyn, E. V. *J. Am. Chem. Soc*. **1998**, *120*, 8533.
5. Harris, D. C. *Quantitative Chemical Analysis*, 4th ed.; Freeman and Co.: New York, 1995; p 352.
6. Kawahara, S.; Uchimaru, T. *Eur. J. Inorg. Chem*. **2001**, 2437.
7. Jelesarov, I.; Bosshard, H. R. *J. Mol. Recog.* **1999**, *12*, 3.
8. Conners, K. A. *Binding Constants*, *The Measurement of Molecular Complex Stability*; John Wiley and Sons: New York, 1987; pp 24-28.
9. Kuzmic, P.; Moss, M. L.; Kofron, J. L.; Rich, D. H. *Anal. Biochem*. **1992**, *205*, 65.
10. (a) Ochiai, E. *J. Chem. Edu*. **1988**, *65*, 943. (b) Bock, C. W.; Katz, A. K.; Glusker, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 3754. (c) Dudev, T.; Lim, C. *J. Am. Chem. Soc.* **2000**, *122*, 11146.
11. The software Dynafit can be obtained at http://www.biokin.com/dynafit/ and the program used for competitive assay was employed.