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Role of Metal Ion in Specific Recognition of Pyrophosphate Ion under Physiological Conditions and Hydrolysis of the Phosphoester Linkage by Alkaline Phosphatase

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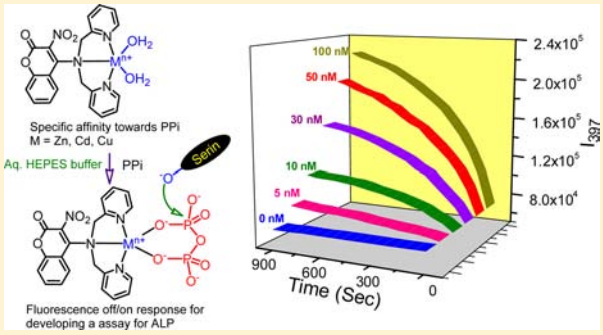
Supporting Information

ABSTRACT: Complexes synthesized from Zn(II), Cu(II), and Cd(II), using a dipicolyl amine derivative (**L**), showed unique specificity toward pyrophosphate ion (PPi or $P_4O_7^{4-}$) among all other common anionic analytes, including different biologically significant phosphate ion (PO_4^{3-}, $H_2PO_4^{2-}$) or phosphate-ion-based nucleotides, such as AMP, ADP, ATP, and CTP. However, the relative affinities of PPi toward these three metal complexes were found to vary and follow the order $K_a^{\mathbf{L.Zn-PPi}} >$ are given in units of ${}_a^{\mathbf{L.Cu-PPi}} \geq K_a^{\mathbf{L.Cd-PPi}}$. Luminescence responses of the receptor **L** were substantial on binding to Zn^{2+} and Cd^{2+}, while relatively a much smaller luminescence response was observed in the presence of Cu^{2+}. Luminescence responses of **L.M**−PPi (**M** is Zn^{2+}, Cd^{2+}, and Cu^{2+}) were further modified on binding to the PPi ion. This could be utilized for quantitative detection of PPi in physiological condition as well as for developing a real time "turn-on" (for **L.Zn** and **L.Cu**) and "turn-off" (for **L.Cd**) fluorescence assay for evaluating the enzymatic activity of alkaline phosphatase (ALP). Experimental results revealed how the subtle differences in the binding affinities between PPi and M in **L.M** (**M** is Zn^{2+}, Cd^{2+}, and Cu^{2+}), could influence the cleavage of the phosphoester linkage in PPi by ALP. The DFT calculations further revealed that the hydrolytic cleavage of the metal ion coordinated phosphoester bond is kinetically faster than that for free PPi and thus, rationalized the observed difference in the cleavage of the phosphoester bond by an important mammalian enzyme such as ALP in the presence of different metal complexes.



INTRODUCTION

Recently, research pertaining to fluorescent chemosensors has received considerable significance, with regard to the design of efficient biomarkers, imaging reagents, and developing appropriate enzymatic assay. Such molecular sensors are also being widely used for the analysis of environmental and biological samples as well as for probing biological processes.[1] Simplicity in the detection process, along with the high sensitivity that one can achieve in the analysis of a desired analyte have provided fluorescence-based receptors and associated methodologies an edge over conventional analytical methods that rely on use of sophisticated instrumentation and involve multistep sample preparation. Furthermore, fluorescent marker or imaging reagents offer the advantages of spatial and temporal resolution, along with in vitro or in vivo analysis.[2]

Specific recognition and quantitative estimation of various biologically important phosphate ions have received considerable attention since these ions play crucial role(s) in various bioenergetic processes. Being the product of ATP hydrolysis under cellular conditions,[3,4] pyrophosphate (PPi) is involved in energy transduction in organisms and in controlling metabolic processes by participation in various enzymatic reactions, e.g., DNA replication.[5] Furthermore, the detection of PPi is important in real-time DNA sequencing method,[6] as well as in cancer research.[7] Patients with chondrocalcinosis, which is a common arthritic condition in which calcium pyrophosphate dehydrate (CPPD) form in articular cartilage have also been shown to have a high level of synovial fluid PPi.[8] Thus, detection and discrimination of PPi is important for evaluating the generation of each of these ions during various biological processes and clarifying its roles in these processes. All these have contributed in the recent surge of interests in developing an improved methodology for the specific recognition and sensitive detection of PPi in physiological condition. However, such an objective (i.e., specific binding and recognition of PPi in aqueous medium) is a challenging one, because of the very high solvation enthalpy (584 kcal mol^{-1}) of PPi in aqueous medium. This high hydration enthalpy adversely influences the effective binding of PPi to a receptor with active hydrogen-

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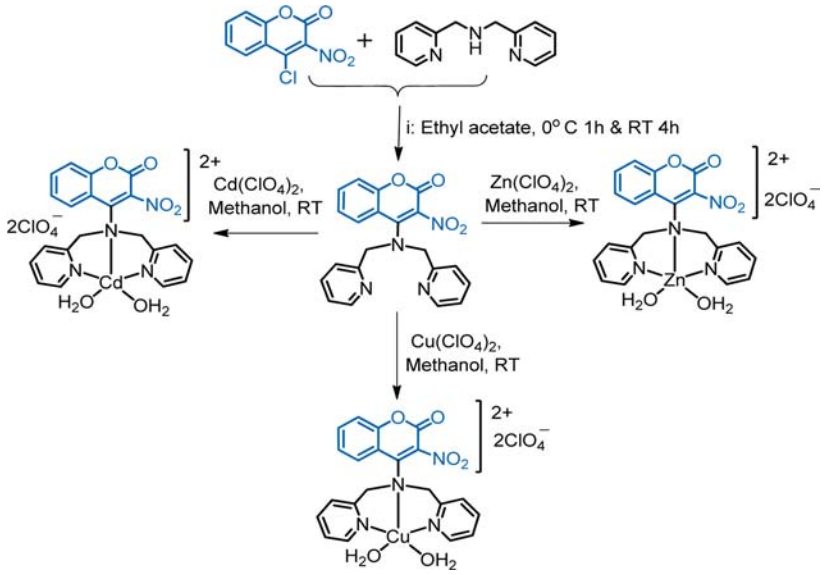
Scheme 1. Molecular Structures and Methodologies Adopted for the Synthesis of L, L.Zn, L.Cd, and L.Cu



bond donor fragment, as the enthalpy change(s) associated with hydrogen-bonded adduct formation fails to compete with this high solvation enthalpy. Apart from this, the presence of other competing anions that have comparable charge density could also interfere in the detection process.[9] As an alternate approach, more recently, researchers have utilized the metal ion-ligand coordination with even higher enthalpy change(s) in aqueous medium for designing receptors for PPi. To date, there have been several reports in the contemporary literature on fluorescent chemosensors for PPi. However, the issue of specificity in the recognition process remains unaddressed in most examples and this limits the application potential of such receptors.[9] Example on specific recognition as well as quantitative detection of PPi over Pi and other phosphate anions such as ATP, AMP, and ADP in aqueous medium is extremely scarce.[10] In one of our recent communication,[11b] we have shown that Zn(II) and Cd(II) complexes could be utilized for achieving this goal and, furthermore, these reagents could even be used for probing the change in concentration of PPi under physiological conditions, as a result of the enzymatic dephosphorylation reaction induced by alkaline phosphatase (ALP). There are only two previous reports available in the literature that talk about probing the enzymatic role that ALP play in effecting a hydrolytic cleavage of the phosphoester linkage by monitoring changes in solution fluorescence as the output signal of a synthetic molecular sensor.[11] In one of these two communications, Schanzeet al. have reported a real time "turn-Off" assay for ALP using the specific coordination of PPi to a Cu^{2+} center with associated changes in fluorescence responses,[11a] while the other communication from the research group authoring this article has shown that DPA complex of Zn(II) or Cd(II) ions could be used for developing a real-time "turn-on" or "turn-off" assay, respectively, for the evaluation of the enzymatic activity of ALP.[11b] ALP is an important enzyme that is common in the mammalian system.[12] Role of ALP is significant for the calcification of cartilage and bone, and it plays a vital role in wide varieties of biological functions. This enzyme is commonly used as a marker for liver function and is also finds a role in laboratory diagnostic.

Thus, the development of any methodology for monitoring the enzymatic activity of ALP is crucial in bioanalytical research, and, in this regard, the use of a molecular sensor capable of probing such an enzymatic process is highly desirable. It has been argued that the deprotonated hydroxyl functionality in the serine moiety, in the vicinity of an active site of ALP, promote the hydrolytic cleavage of the phosphoester linkages of various molecules such as alkaloids, nucleotides, and proteins through a nucleophilic attack on phosphorus.[13] A recent report also suggests certain interactions between the Zn^{2+} site and a nonbridging phosphate ester oxygen atom accounts for the large rate enhancement observed for ALP-catalyzed phosphate monoester hydrolysis. However, the role of the metal ions, bound to a phosphate moiety, in achieving the phosphoester bond cleavage in PPi by ALP is not well-understood. In this article, we have reported results on the relative affinity of the respective DPA-based (**DPA-M**, where **M** = Zn^{2+}, Cd^{2+}, Cu^{2+}) metal complex toward PPi and the influence of the subtle difference in such affinity on the efficiency of the cleavage of the phosphoester linkage of PPi through the nucleophilic attack of the ALP. These results have provided us the crucial clue about the possibility to delineate the reaction of the metal ion coordinated or free PPi with ALP in the phosphoester bond cleavage reaction and thus, developing a better insight in understanding the enzymatic activity of ALP. Our presumption was further corroborated by the results of the density functional theory (DFT) studies.

EXPERIMENTAL SECTION

Materials and Method. Chemicals such as 3-nitro-4-choloro coumarin, dipicolyl amine, $Hg(ClO_4)_2$, $Cd(ClO_4)_2$, $Zn(ClO_4)_2$, $Ni(ClO_4)_2$, $Co(ClO_4)_2$, $Pb(ClO_4)_2$, $Fe(ClO_4)_2$, $Cu(ClO_4)_2$, $Cr(ClO_4)_2$, and different nucleotides (adenosine 5′-monophosphate monohydrate, adenosine 5′-diphosphate sodium salt, adenosine 5′-triphosphate disodium hydrate, cytidine 5′-triphosphate disodium salt hydrate), and alkaline phosphatase (ALP) were obtained from Sigma–Aldrich and were used as received without further purification. Other salts, such as NaF, NaI, NaBr, NaOAc, NaCl, NaH_2PO_4, $Na_4P_2O_7$, Na_2SO_4, and $NaNO_3$ and all of the other reagents used were of reagent grade (S. D. Fine Chemical, India) and were used as received. High-performance liquid chromatography (HPLC)-grade water (Fisher Scientific) and acetonitrile (Merck India) was used as a solvent. Ethyl acetate and methanol, which were used for different synthetic procedures, were purified through distillation following standard

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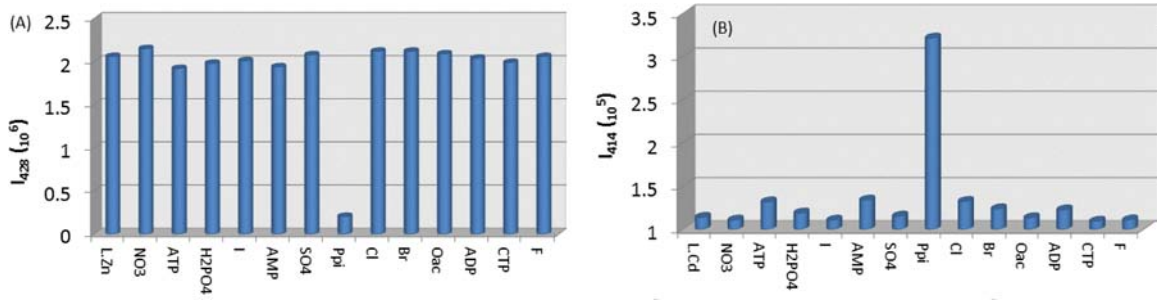

Figure 1. Luminescence responses of (A) **L.Zn** (2.0×10^{-5} M) and (B) **L.Cd** (2.0×10^{-5} M) in aqueous 0.01 mmol HEPES buffer (pH 7.4) medium upon the addition of sodium salt of various anionic analytes and nucleotides (2.0×10^{-4} M) with λ_{Mon} = 428 nm (for **L.Zn**, λ_{Ext} = 328 nm) and λ_{Mon} = 414 nm (for **L.Cd**, λ_{ext} = 328 nm).

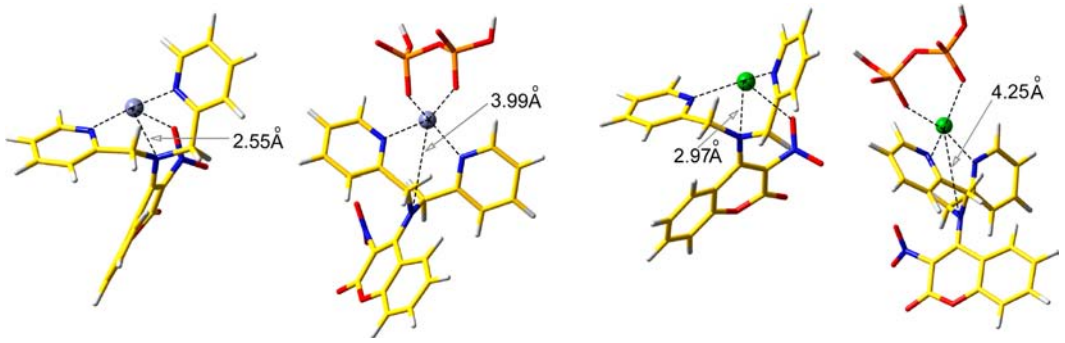

Figure 2. Energy optimized structures for **L.Zn**, **L.Zn**–PPi, **L.Cd**, and **L.Cd**–PPi obtained at the B3LYP/[LANL2DZ-ECP + 6-31G*] level in the aqueous phase. (Atom color codes: red = oxygen, white = hydrogen, orange = phosphorus, yellow = carbon, blue = nitrogen, violet blue = zinc, and green = cadmium.)

procedures, prior to use. Synthesis of **L**, **L.Zn**, and **L.Cd** are reported in our earlier communication.[11b] Analytical and spectroscopic data of these compounds, used for the present study, confirmed the desired purity of these compounds.

Instrumentation. Microanalysis (C, H, N) were performed using a Perkin–Elmer 4100 elemental analyzer. Fourier transform infrared (FTIR) spectra were recorded as KBr pellets using a Perkin–Elmer Spectra GX 2000 spectrometer. ^{1}H and ^{31}P NMR spectra were recorded on Bruker 200 MHz (Avance-DPX 200)/500 MHz (Bruker Avance II 500) FTNMR system using CD_3CN as the solvent and tetramethyl silane (TMS) or H_3PO_4 as an internal standard. Electronic spectra were recorded with Cary–Varian ultraviolet–visible–near-infrared (UV-vis-NIR) spectrophotometer. Fluorescence spectra were recorded using Fluorolog (Horiba Jobin–Yvon) or Edinburgh F920 (Edinburgh Instruments) fluorescence spectrometer. ESI-MS measurements were carried out on Waters QTof-Micro instrument.

Synthesis of L.Cu. **L** (100 mg, 0.257 mmol) was dissolved in 20 mL of methanol. $Cu(ClO_4)_2 \cdot 6H_2O$ (145 mg, 0.391 mmol) solution in 5 mL of HPLC-grade water was added to this solution of **L** in a dropwise manner. The resultant solution was allowed to stir for 4 h at room temperature. A bluish white precipitate appeared which was filtered off. The residue was washed thoroughly with cold methanol and methanol–water (4:1, v/v). Then the washed residue was dried in air for isolating desired metal complex (**L.Cu**) in pure form. Yield: 77.3 mg, 46.3%. IR (KBr) ν_{max} (cm^{-1}): 3437, 3049, 2984, 2684, 2362, 1655, 1549, 1503, 1457, 1340, 1285, 1229, 1186, 1093, 909, 861, 772, 621. ESI-MS (m/z): 587.66 ($M^+ + ClO_4^- + 2H_2O$, 65%), 687.70 ($M^+ + 2ClO_4^- + 2H_2O$, 50%). Elemental analysis: $C_{21}H_{20}Cl_2N_4O_{14}Cu \cdot 2H_2O$; calculated C 36.72, H 2.94, N 8.16; found C 36.7, H 2.91, N 8.13.

Computational Methodology. All the geometries of the stationary points were fully optimized in aqueous phase at the B3LYP level of theory.[14] All main group atoms were used with 6-31G* basis set,[15] and LANL2DZ ECP[16] was employed for the Zn and Cd (transition metals). Polarizable continuum solvation model (PCM) was also employed in the calculations using the integral equation formalism variant (IEF-PCM).[17] The stationary points were fully characterized by frequency calculations in order to verify that the transition structures have one, and only one, imaginary frequency. The minima were characterized by no imaginary frequency in the calculations. To verify each saddle point connects two minima, intrinsic reaction coordinate (IRC) calculations of transition states have been performed in both directions, that is, by following the eigenvectors associated to the unique negative eigenvalue of the Hessian matrix, using the González and Schlegel integration method.[18] All calculations were performed with Gaussian 09 suite program.[19]

Luminescence Study. A 1.0×10^{-4} M solution of compound **L.M** (**M** = Zn^{2+}, Cd^{2+}, Cu^{2+}) (Scheme 1) in an aqueous buffer (0.01 M HEPES buffer, pH 7.4) was prepared and stored in darkness. This solution was used for further spectroscopic studies after appropriate dilution. 1.0×10^{-3} M solutions of inorganic salt of the respective anions and different nucleotides (ATP, ADP, CTP, AMP) were prepared in an aqueous buffer (0.01 M HEPES buffer, pH 7.4) medium and each solution was stored in an inert atmosphere. For scanning fluorescence responses of **L.M** toward different anionic analytes, the concentration of **L.M** was adjusted to an effective final concentration of 2.0×10^{-5} M, while that for respective anions was set at 2.0×10^{-4} M. For the fluorescence titration studies, the effective concentration of **L.M** used was adjusted to 2.0×10^{-5} M; while the final $[Na_4P_2O_7]$ were varied over ranges of $0–6.0 \times 10^{-5}$ M for studies with **L.Zn**, $0–1.0 \times 10^{-4}$ M for studies with **L.Cd**, and $0–8.5 \times 10^{-5}$ for studies with **L.Cu**. $Na_4P_2O_7$ solution was introduced in an incremental fashion during the luminescence titrations and corresponding spectra were recorded at 298 K. The association constants and binding stoichiometry of **L.M** with $P_2O_7^{4-}$ were determined using a Benesi–Hildebrand (eq 1) plot:

$$\frac{1}{F - F_0} = \frac{1}{K(F_{max} - F_0)[A_n^{n-}]} + \frac{1}{F_{max} - F_0} \quad (1)$$

where F_0 is the luminescence intensity of **L.M** at a particular wavelength, F is the luminescence intensity obtained with externally

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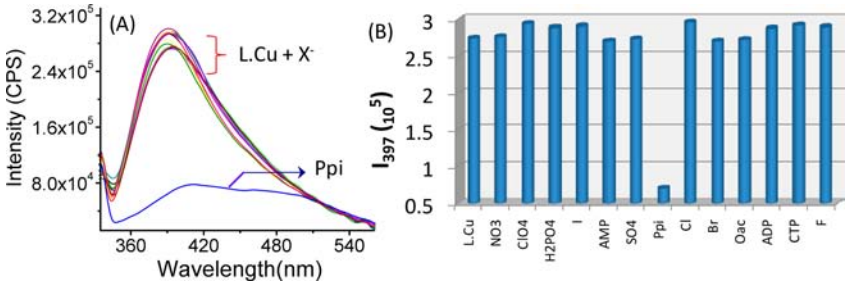

Figure 3. Fluorescence scanning of **L.Cu** (2.0×10^{-5} M) with sodium salts of various anions and common nucleotides (10 mol equiv) ($X^- = F^-$, $H_2PO_4^-$, Cl^-, Br^-, I^-, SO_4^{2-}, NO_3^-, CH_3COO^-, CTP, ClO_4^-, ADP, AMP) in 0.01 M aqueous HEPES buffer (pH = 7.4) medium using λ_{Ext} = 320 nm. (B) Luminescence response of **L.Cu** (2.0×10^{-5} M) in the absence and presence of sodium salt of different anions and nucleotides (2.0×10^{-4} M) in 0.01 M aqueous HEPES buffer (pH 7.4) medium with λ_{Mon} = 397 (for λ_{Ext} = 320 nm).

added $P_2O_7^{4-}$ (PPi) at that particular wavelength, F_{max} is the intensity at the saturation point, K is the association constant (M^{-1}), and $[A_n^{n-}]$ is the concentration of $P_2O_7^{4-}$ ($[P_2O_7^{4-}]$) that was added externally.

RESULTS AND DISCUSSION

The coumarin derivative (**L**) and two previously reported compounds, **L.Zn** and **L.Cd** were synthesized following the reported procedure (see Scheme 1). The new complex, **L.Cu**, was synthesized by reacting an aqueous solution of $Cu(ClO_4)_2$ with a methanolic solution of **L**. All the complexes were isolated as pure solid and were characterized using various analytical and spectroscopic techniques. All analytical and spectroscopic data agreed well with the proposed formulations for these compounds. Transition-metal ions such as Zn^{2+}, Cd^{2+}, and Cu^{2+} usually have a distinct affinity toward phosphate anions. In our previous report, we had shown that presynthesized complexes **L.Zn** and **L.Cd** showed unique specificity toward PPi in the presence of all other common anionic analytes, including phosphate (Pi) as well as other phosphate ions derivatives (AMP, ADP, ATP, and CTP) in a 0.01 M aqueous HEPES buffer medium (pH 7.4).

As illustrated in Figure 1, a significant switch-off luminescence response was observed for **L.Zn** at λ_{max} = 428 nm upon the addition of NaPPi (10 mol equiv). Analogous studies with **L.Cd** revealed a prominent switch-on luminescence response at 414 nm upon the addition of NaPPi (10 mol equiv). The reason for such a switch on/off emission response was not understood earlier, and we could address this specific issue using results obtained from the theoretical studies. A close comparison of the energy optimized structures for **L.M** (**M** = Zn^{2+} or Cd^{2+}) and **L.M**–PPi (Figure 2) clearly revealed (vide infra) that the $M-N_{tertiary\ amine}$ bond in **L.M** becomes much longer and thus weaker in **L.M**–PPi. This weakens the interrupted PET process in **L.M**–PPi, which was otherwise operational in **L.M**.

Influence of the metal ion-anion coordination on fluorescence responses has been reported earlier by different researchers;[9f,10b,g] however, the basis of explaining such an observation was, at best, speculative. Results of our theoretical studies clearly provided the necessary insight in explaining such a fluorescence response. For the Cu^{2+} ion, presumably two opposing influences, such as interrupted PET and the efficient fluorescence quenching processes due to paramagnetic effect, could have canceled out each other. More importantly, efficient hydration of Cu^{2+} in an aqueous medium could have had an adverse influence on the coordinative binding of Cu^{2+} to **L**. All these presumably had contributed to the insignificant change in the observed emission spectra on binding of Cu^{2+} to **L** in an aqueous medium. These observations prompted us to synthesize the complex **L.Cu** from a predominantly non-aqueous medium.

Emission response of the solution of the presynthesized complex **L.Cu** was examined in the absence and presence of various common anions, including Pi, PPi, and other common nucleotides such as ATP, ADP, AMP, and CTP. Except PPi, all other anions failed to induce any detectable change in the emission spectral pattern for **L.Cu** (see Figure 3). Thus, all three metal complexes (**L.Zn**, **L.Cd**, and **L.Cu**) exhibited specific luminescence response toward PPi over other anions including Pi and other common nucleotides, such as ATP, ADP, AMP, and CTP. The relative affinities of **L.Zn** and **L.Cd** toward PPi were found to be 3.18×10^5 M^{-1} and 2.68×10^4 M^{-1}, respectively, based on the results of a Benesi–Hildebrand (B–H) plot utilizing the data from fluorescence titrations.[20]

Binding affinity of PPi toward **L.Cu** was also evaluated using data obtained from systematic fluorescence titration for a fixed [**L.Cu**] of 2.0×10^{-5} M with varying [PPi] (0–8.5×10^{-5} M) in 0.01 M aqueous HEPES buffer (pH 7.4) medium (Figure 4A). A binding constant of 3.59×10^4 M^{-1} was obtained from

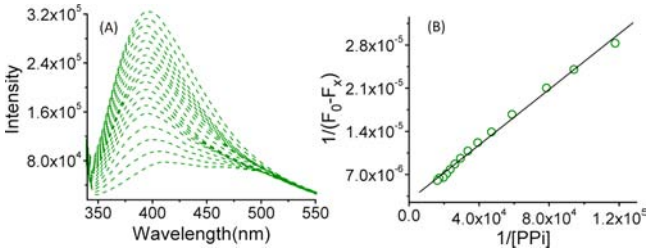

Figure 4. (A) Emission titration profile of **L.Cu** (2.0×10^{-5} M) with varying [NaPPi] (0–8.5×10^{-5} M) in a 0.01 M aqueous HEPES buffer (pH 7.4) medium, using λ_{Ext} = 320 nm. (B) Benesi–Hildebrand (B–H) plot of data obtained from emission titration; the good linear fit of the B–H plot confirmed the 1:1 binding stoichiometry.

the B–H plot. The good linear fit of the $1/(F_0 - F_x)$ vs $1/[PPi]$ plot confirmed a 1:1 binding stoichiometry and thus, the formation of **L.Cu**-PPi in a buffer medium having pH 7.4 (see Figure 4B). This stoichiometry was further confirmed from the results of the ESI-MS analysis (see SI Figure 3 in the Supporting Information).

Phosphoryl transfer reactions play a fundamental role in a wide range of biological processes including basic metabolism, energy transduction, gene expression, and cell signaling. Uncatalyzed phosphoryl transfer reactions are reported to be extremely slow, but enzymes can provide rate enhancements of

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$>10^{20}$ fold to allow these reactions to occur on a biologically relevant time scale.[21] It has been argued that the enzymatic rate enhancements arise from preferential stabilization of the transition state, relative to the ground state.[22] Thus, the understanding of such process that accounts for the stabilization of the transition state is important to design such reactions. Among various important enzymes, ALP is one of the most significant one. ALP activity in human serum has significance in the diagnosis of several crucial diseases, particularly those involving the liver and bone.[23,24] However, this enzyme is routinely applied as a marker for liver function, and it is used extensively in laboratories for diagnostic purposes. However, in many instances, its reactivity and thus its biological relevance are poorly understood. All these have necessitated the development of an efficient strategy for monitoring the enzymatic activity of ALP and is a challenging issue in bioanalytical research.[12a,13a,24] In this context, not only we have explored the possibility of using these presynthesized PPi specific metal ion complexes (**L.Zn**, **L.Cd**, and **L.Cu**) as a bioanalytical tool to buildup both real-time fluorescence based "turn-on" and "turn-off" assays for the evolution of enzymatic activity of ALP, but also to develop a better insight on the mechanistic pathway for the phosphoester hydrolysis reaction.

Results of the earlier studies described above clearly revealed that these three reagents could be used for specific recognition and quantitative estimation of the [PPi] in physiological conditions. This led us to explore the possibility of probing the effective concentration of PPi under physiological conditions, because of the hydrolytic cleavage of the phosphoester bond induced by ALP. Results of the studies described above clearly reveal that fluorescence response of the solution of **L.M** (**M** = Zn^{2+}, Cd^{2+}, and Cu^{2+}) in aqueous HEPES buffer (pH 7.4) got altered only on specific binding to PPi; while $H_2PO_4^-$ or other phosphate ions derivatives failed to induce any such significant changes.

We exploited this for probing the changing concentration of PPi due to the hydrolytic cleavage of PPi to inorganic phosphate ion by ALP and thus eventually developing an assay for the enzymatic phosphoester cleavage reaction by ALP. To check such a possibility, we monitored the fluorescence responses at different monitoring wavelength (λ_{Ext} = 328 or 320 nm) as a function of time and [ALP] for three sets of following solutions: solution (a), 20 μM **L.Zn** + 40 μM NaPPi + varying [ALP]; solution (b), 20 μM **L.Cd** + 140 μM NaPPi and solution + varying [ALP]; and solution (c), 20 μM **L.Cu** + 40 μM NaPPi + varying [ALP]. It is worth mentioning that solutions were incubated at 37 °C for 10 min prior to the addition of any ALP. After the addition of different concentration of ALP (bovine intestinal mucosa) to the different assays, we had recorded emission intensity at 428 nm for assay having **L.Zn** (λ_{Ext} = 328 nm), at 414 nm for assay having **L.Cd** (λ_{Ext} = 328 nm) and at 397 nm for assay having **L.Cu** (λ_{Ext} = 320 nm) as a function of time with varying concentration of ALP (see Figure 5, as well as SI Figure 7 in the Supporting Information). Results for **L.Zn** and **L.Cd** were briefly discussed in our previous communication. Results obtained for **L.Cu** are discussed in detail in the following section. ALP is known to catalyze the hydrolytic cleavage of the phosphoester bond in PPi. A gradual increase in emission intensity of the assay having **L.Zn** (λ_{Mon} = 428 nm) (see SI Figure 7A in the Supporting Information) or **L.Cu** (λ_{Mon} = 397 nm) (see Figure 5) and a decrease in the emission intensity of the assay having **L.Cd** was observed with increasing [ALP] (see

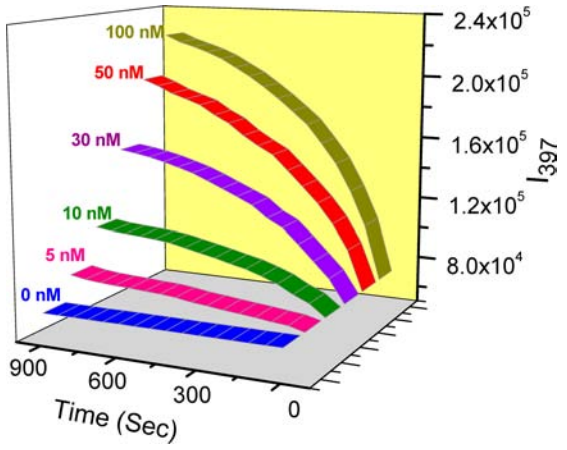

Figure 5. Representative ribbon diagram of the real-time ALP assay having 20 μM **L.Cu** + 140 μM NaPPi (λ_{Ext} = 320 nm, λ_{Mon} = 397 nm) in 0.01 M HEPES buffer medium, pH 7.4.

SI Figure 7B in the Supporting Information). More importantly, the extent of changes in emission intensity for different assay at their respective monitoring wavelength was found to increase with time for a certain [ALP] value.

Thus, this study clearly revealed that these three DPA-based PPi-specific metal complexes could be utilized for developing either real-time "Turn-On" (assays a and c) and "Turn-Off" (assay b) fluorescence assay for probing the enzymatic activity of ALP.

Rate constants of hydrolytic cleavage of the phosphoester bond were evaluated from the data obtained from the time-dependent studies (see Figure 6, as well as SI Figures 8 and 9 in the Supporting Information) for a fixed [ALP] and are summarized in Table 1. These results, described above, suggest that an effective fluorescent assay could be developed for probing the enzymatic activity of the ALP.

Close scrutiny and comparison of the relative binding affinities of PPi toward these three complexes reveal that the trend in relative binding affinity and trend in rate for the hydrolytic cleavage reaction are similar (Table 1). This tends to suggest that PPi bound to a metal ion in **L.M** is undergoing hydrolysis and not the free PPi ion that could exist in equilibrium. However, the possibility of hydrolytic dephosphorylation process involving the free PPi that could be present in the reaction medium (and, thus, the influence of the slightly depleted [PPi] on the equilibrium for the formation of **L.M**–PPi) could not be completely ruled out.

It has been argued that the active site of the each subunit in ALP contains two Zn^{2+} ions and an arginine residue that adopt appropriate orientation to interact with the substrate and provides the serine alkoxide moiety of ALP to displace the inorganic phosphate (Pi) as the leaving group in the first step of the reaction to produce a covalent enzyme–phosphate intermediate (see Scheme 2A).[13b,23,24b]

This intermediate is presumed to be hydrolyzed in the second step of the reaction (Scheme 2B).[24b] For the present study, to delineate the involvement of either the free or metal-bound PPi in cleavage reaction induced by ALP, a detailed density functional calculations (DFT) were carried out. This was also crucial in developing a better insight in understanding how the subtle differences in binding affinities between **L.M** and PPi can influence the catalytic cleavage of the phosphoester linkage.

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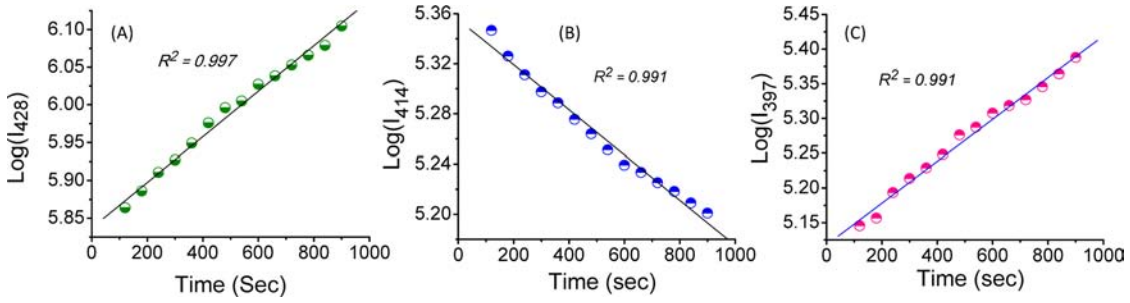
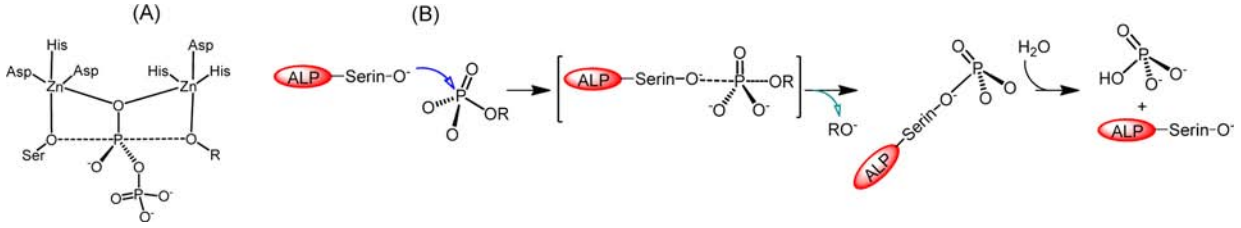


Figure 6. Kinetic analyses (plot of log(I) vs time, where I is the fluorescence intensity at the respective monitoring wavelength) of different assays having (A) 20 μM **L.Zn** + 40 μM NaPPi + 100 nM ALP (λ_{Ext} = 328 nm, λ_{Mon} = 428 nm), (B) 20 μM **L.Cd** + 140 μM NaPPi + 100 nM ALP (λ_{Ext} = 328 nm, λ_{Mon} = 414 nm), and (C) 20 μM **L.Cu** + 140 μM NaPPi + 100 nM ALP (λ_{Ext} = 320 nm, λ_{Mon} = 397 nm) in 0.01 M HEPES buffer medium at pH 7.4 and 37 °C.

Table 1. Correlation of the Rate of Hydrolysis of Different Assays and Corresponding Association Constants of Complexation between the Metal Complexes and PPi

assay system	λ_{Ext} and λ_{Mon}	$K_{ass}^{L.M-PPi}$ (M^{-1})	rate constant
(a) 20 μM **L.Zn** + 40 μM NaPPi+ 100 nM ALP	λ_{Ext} = 328, λ_{Mon} = 428	(3.18 ± 0.08) × 10^5 (**M** = Zn^{2+})	34 ± 0.8 s^{-1} M^{-1}
(b) 20 μM **L.Cd** + 140 μM NaPPi + 100 nM ALP	λ_{Ext} = 328, λ_{Mon} = 414	(2.68 ± 0.06) × 10^4 (**M** = Cd^{2+})	3.5 ± 0.02 s^{-1} M^{-1}
(c) 20 μM **L.Cu** + 140 μM NaPPi + 100 nM ALP	λ_{Ext} = 320, λ_{Mon} = 397	(3.59 ± 0.06) × 10^4 (**M** = Cu^{2+})	5.8 ± 0.03 s^{-1} M^{-1}

Scheme 2. (A) Transition-State Model for Phosphoryl Transfer Catalyzed by ALP Based on the Structure with a Bound-Metal-Based Receptor;[13b,23,24b] (B) Probable Mechanism of the Phosphate Monoester Hydrolysis by ALP through a Loose Transition State



COMPUTATIONAL RESULTS

The binding affinity of **L.Zn** toward PPi was found to be substantially higher (Table 1) than that for **L.Cd** and **L.Cu**, whereas that for **L.Cu** was marginally higher than that for **L.Cd**. Thus, we have performed quantum chemical calculations in order to examine the transition states for the phosphoester cleavage reaction of PPi by ALP in the absence and presence of **L.Zn** and **L.Cd** using computational studies. Also, zinc and cadmium belong to the same group in the periodic table, so our studies with **L.Zn** and **L.Cd** would enable us to examine the variation in their reactivity toward the cleavage of phosphoester linkage of PPi.

The ALP was modeled with the serine moiety in the DFT calculations. To understand the influence of metal ions for the phosphoester bond cleavage reaction, the initial study was performed without the involvement of ligand and metal ions. The serine unit of ALP would act as a nucleophile to cleave the phosphoester bond of pyrophosphate. The potential energy surface for the attack of serine on the P atom of pyrophosphate is given in Figure 7.

The calculated activation barrier for the cleavage of the phosphoester bond was evaluated as 25.6 kcal/mol with the B3LYP level of theory. The product complex was slightly endothermic in nature, with respect to reactants. This result indicated that the cleavage of phosphoester bond of free pyrophosphate was energetically demanding and, hence, the reaction needed to be catalyzed to facilitate the process. The similar phosphoester bond cleavage reaction of pyrophospate was examined with the **L.Zn** and **L.Cd**. As mentioned earlier, energy-optimized structures for **L.M**–PPi (M is Zn^{2+} or Cd^{2+}) suggested that the coordination of PPi to the respective metal ion in **L.M** caused a substantial elongation of the M–$N_{tertiary\ amine}$ bond, which resulted in the generation of a tetrahedral geometry for the Zn^{2+} or Cd^{2+} center (recall Figure 2). This proposition was further substantiated by the fluorescence "off" and "on" responses, respectively, for Zn^{2+} and Cd^{2+} complexes. The approach of serine moiety toward the P atom of pyrophosphate was carried out with the **L.Zn**–PPi (see Figure 8).

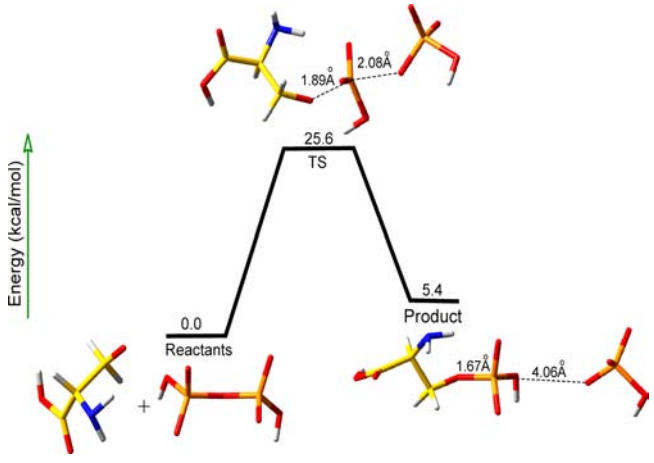


Figure 7. Potential energy surface and the corresponding geometries for phosphoester cleavage of free pyrophosphate at B3LYP/6-31G* level in aqueous phase. Relative free energies are given in units of kcal/mol, and distances are given in units of Å. (Atom color code: red = oxygen, white = hydrogen, orange = phosphorus, yellow = carbon, and blue = nitrogen.)

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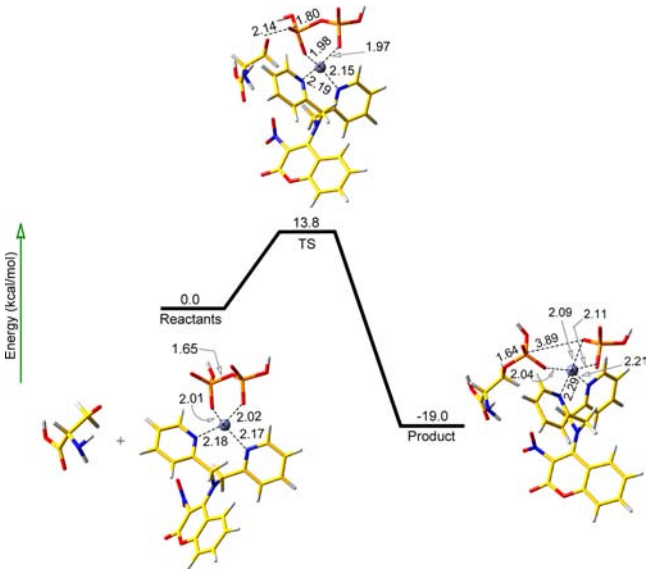

Figure 8. Potential energy surface and the corresponding geometries for phosphoester cleavage of pyrophosphate in the presence of **L.Zn** at the B3LYP/[LANL2DZ-ECP + 6-31G*] level in the aqueous phase. Relative free energies are given in units of kcal/mol, and distances are given in units of Å. (Atom color code: red = oxygen, white = hydrogen, orange = phosphorus, yellow = carbon, blue = nitrogen, and violet blue = zinc.)

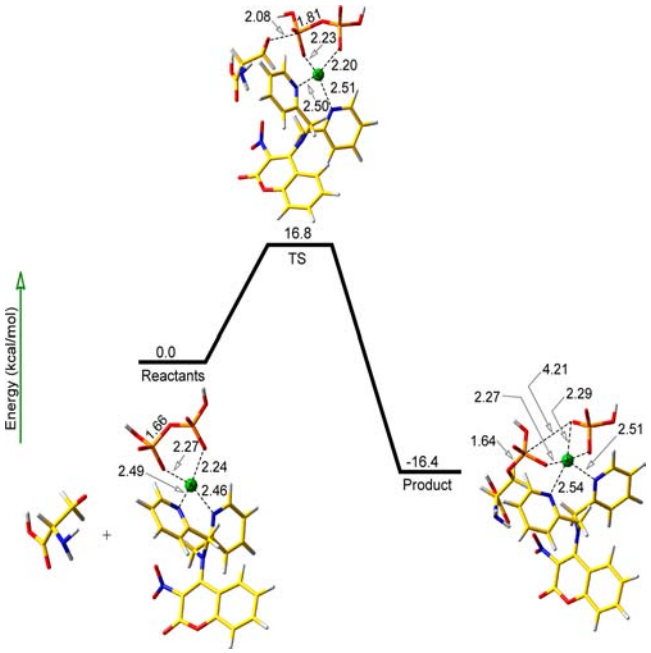

Figure 9. Potential energy surface and the corresponding geometries for phosphoester cleavage of PPi in the presence of **L.Cd** at the B3LYP/[LANL2DZ-ECP + 6-31G*] level in the aqueous phase; Relative free energies are given in units of kcal/mol, and distances are given in units of Å. (Atom color code: red = oxygen, white = hydrogen, orange = phosphorus, yellow = carbon, blue = nitrogen, and green = cadmium.)

The fifth coordination with one of the tertiary nitrogen atoms of the DPA moiety of **L** was not achieved in this case, because of the strain caused upon complexation with the pyrophosphate group. The activation barrier calculated for the attack of serine moiety to the **L.Zn**−PPi was estimated as 13.8 kcal/mol, which was much lower than that was evaluated for unbound phosphoester bond cleavage reaction. In the transition state involving **L.Zn**−PPi and serine moiety, the Zn^{2+} ion was found to form a more ordered tetrahedral complex with the **L** and pyrophosphate.

Importantly, the complex was also found to become much tighter, compared to the reactant complex (Figure 8). The coordinating distances of **L** and PPi with Zn^{2+} ion were found to relatively shorter in the transition state, compared to those in **L.Zn**−PPi (see Figure 8). The product complex was highly exothermic in nature on the potential energy surface. These results suggest that the Zn^{2+}-catalyzed phosphoester bond cleavage reaction of PPi is energetically more preferred than the unbound PPi one.

Analogous studies were further extended with **L.Cd**. Analogous to **L.Zn**−PPi, the Cd^{2+} ion in **L.Cd**−PPi adopted a tetrahedral geometry (see Figure 9). Energy-optimized structures revealed that Cd^{2+} formed a less tighter complex, compared to the corresponding Zn^{2+} complex, which is well supported by the fact that the ionic size of Cd^{2+}(0.95 Å) was known to be relatively larger than Zn^{2+}(0.74 Å) (compare Figures 8 and 9). The activation barrier calculated for the phosphoester bond cleavage reaction with the Cd^{2+} was 16.8 kcal/mol, which was higher than that was evaluated for the analogous Zn^{2+} complex. However, this activation barrier was much lower than that for the phosphoester bond cleavage reaction of unbound PPi. The calculated distances between the coordinating atoms of **L** and Cd^{2+} ion were found to relatively longer in the transition state, compared to corresponding bound form (**L.Cd**−PPi), which was contrary to the Zn^{2+} coordinated case and presumably was responsible for the higher activation barrier. These results are in good agreement with the observed rates for the phosphoester bond cleavage reaction of PPi (see Table 1 and SI Figures 8 and 9 in the Supporting Information).

CONCLUSIONS

In the present study, we could demonstrate that, along with **L.Zn** and **L.Cd**, **L.Cu** also could be used to develop a fluorescence-based assay for probing the enzymatic cleavage of the phosphoester bond under physiological conditions, which has significance in developing an appropriate diagnostic tool for ALP. More importantly, the results of our experimental studies could confirm that relative differences in binding affinities of **L.M** (**M** = Zn^{2+}, Cd^{2+}, and Cu^{2+}) toward PPi followed the similar order of reactivity toward the hydrolytic cleavage of the phosphoester bond in PPi by ALP. This suggested that it was the bound PPi that participated in the phosphoester bond cleavage reaction and not the free PPi that could exist in equilibrium. The density functional theory (DFT) calculations showed that the hydrolytic cleavage of the metal-ion-bound phosphoester bond was kinetically faster than that for free PPi. Furthermore, the efficacy of Zn^{2+} catalyzed hydrolytic cleavage of the phosphoester bond, compared to the Cd^{2+}-catalyzed case, is also borne out by the DFT calculations.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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