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Dizinc Enzyme Model/Complexometric Indicator Pairs in Indicator Displacement Assays for Inorganic Phosphates under Physiological Conditions

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A dizinc phosphohydrolase enzyme model complex employing the dinucleating ligand 2,6-bis-[(bis-pyridin-2-ylmethyl-amino)methyl]-4-methylphenol (**L1**) was tested for binding to a series of 11 commercially available complexometric indicators in aqueous *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 7.4, with the aim of determining the applicability of these indicators in indicator displacement assays (IDAs) under physiological conditions. Dissociation constants (K_d) were determined for 11 indicator–Zn_2**L1** complexes, spanning 2 orders of magnitude from 2.8×10^{-4} M (alizarin red S) to 2.7×10^{-6} M (bromo pyrogallol red). Phosphate and pyrophosphate were tested for their ability to displace bound indicator and produce a detectable colorimetric response. Three indicators (bromo pyrogallol red, mordant blue 9, and zincon) complex to Zn_2**L1** to form an indicator displacement assay selective for pyrophosphate over phosphate. Because selection of an indicator/analyte pair having appropriate relative K_d values is critical for their successful application in IDAs, the binding data for these 11 indicators should assist their extension to IDAs for other analytes.

Introduction

Inorganic phosphates (IPs) and organophosphorus species (OPs) play a number of key roles in physiology and pathophysiology. The fidelity of genetic replication relies on robust phosphoester linkages that make up DNA and RNA backbones. Even the simplest inorganic phosphorus oxyanion, phosphate, is involved in innumerable biological processes. Heightened phosphate levels (hyperphosphatemia) lead to renal failure, and phosphate deficiency (hypophosphatemia) is responsible for hyperthyroidism and rickets.[1] Phosphate is also a biological buffer, notably intracellularly, in the urinary tract, and in saliva where it buffers against bacterial acid-induced tooth decay.[2,3] Pyrophosphate ($P_2O_7^{4-}$, often abbreviated PP_i) is the product of ATP hydrolysis, the hallmark reaction of bioenergetics and metabolism, which regulates numerous enzymatic reactions.[4] Phosphorylation is an important post-translational modification affecting protein structure and function through allosteric activation.[5,6] Phospholipids are also key cell membrane components. Many senescence theories and associated preventative medicines focus on phospholipid bilayer deterioration as a primary cause of aging.[7−9] Pathophysiological responses can also result from xenobiotic OPs. Organophosphorus pesticides and the nerve agents of chemical warfare are among the most toxic substances ever isolated.[10,11] These OPs induce toxic effects primarily by phosphorylation of a serine residue within the active site of acetylcholinesterase, leading to excess cholinergic stimulation within the synaptic cleft.[12]

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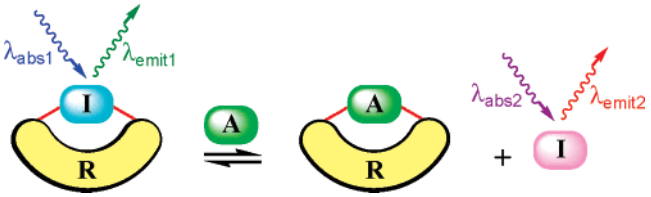
Scheme 1. Generalized Representation of the Signal Transduction Mechanism for IDAs; I = Indicator, A = Analyte, R = Receptor[a]

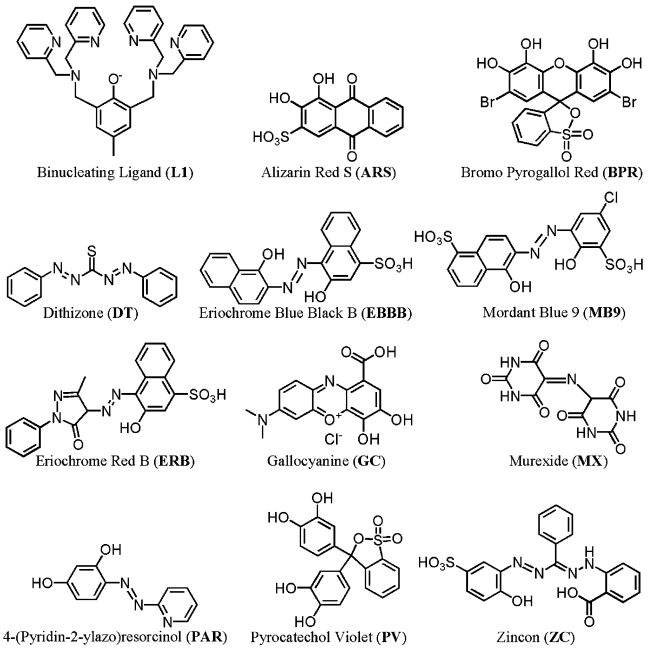


[a] Emission or absorption intensity or wavelength maxima may change between bound and unbound states.

Environmentally, phosphate is an essential soil nutrient for healthy plant growth and is a common component in agricultural supplementation.[13−15]

The multifaceted role of inorganic phosphates and their phosphoester derivatives in biology and the environment has led researchers to develop various means of detecting these species. UV−vis and fluorescence-based optical methods have been particularly pursued due to the convenient visual ("by eye") detection provided by colorimetric assays and the high sensitivity of fluorescence methods. The indicator displacement assay (IDA) strategy is a simple and increasingly popular approach to optical detection. In this strategy (Scheme 1), a receptor is designed to bind a target analyte with a desired affinity, and an indicator is selected that has a weaker affinity for the receptor than the target analyte does. The indicator must also exhibit a notable optical change upon displacement, thereby providing a means of assessing analyte presence and quantifying concentration spectroscopically. The IDA strategy has emerged as an attractive alternative to covalently tethered receptor−reporter constructs due to synthetic simplification and applicability of a single receptor to differentiation of various analytes.[16,17] Anslyn has pioneered IDAs for amino acids[18,19] and carboxylates present in beverages,[20−25] for example. Other groups developing IDAs for similar analytes have focused on metal complexes as receptors.[26−31] Fluorescent IDAs for detecting nitric oxide (NO)[32−35] exhibiting emission enhancement upon displacement of fluorogenic ligands from quenching transition-metal centers have proven effective under physiological conditions and have even been used to image NO synthesis by living cells.[34] IDAs for phosphate derivatives, the analytes of interest in the current study, have also been explored.[30,36] Neutral organophosphorus nerve agent simulants can be detected via metal ion displacement from fluorogenic 2,6-bis(1′-methylbenzimidazolyl)pyridine ligands,[37] while IDAs for anionic phosphate derivatives[38,39] often utilize dizinc complexes as the receptor module. Dizinc phosphohydrolase model complexes supported by **L1** (Chart 1) have proven to be particularly useful receptors for IDAs.[39−46] Chemosensors

Chart 1. Dinucleating Ligand and Complexometric Dyes Used in the Current Study[a]



[a] Only one protonation state and resonance structure is shown in each case. Ligand **L1** is shown in the phenolate form present in the Zn_2**L1** complex.

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using the Zn_2**L1** receptor or a derivative thereof have demonstrated stability in vivo, as exemplified by a recent application to imaging bacterial infection in live mice.[38] Complexometric indicators are well-known for their dramatic color changes in response to metal ions and are widely used for metal ion detection. As such, they are also obvious candidates to serve as displaceable chromophores from metal complex receptors in IDAs. Preliminary studies in this vein have used pyrocatechol violet (PV) or other indicator molecules binding the Zn_2**L1** center via a catecholate moiety[47] as the displaced indicator.[48−50] Although the interaction of complexometric indicators with single metal ions in solution has been well-studied,[51−57] knowledge of analogous binding interactions between these indicators and bimetallic centers remains incomplete. The current work examines in detail the binding of 11 well-known, commercially available complexometric indicators (Chart 1) with the dizinc phosphohydrolase model compound Zn_2**L1**. Subsequent displacement of the indicators by phosphate and pyrophosphate is explored to test the viability of each indicator as a displaceable reporter. These data should assist in the rational design of future indicator displacement sensors.

Results and Discussion

The ligand **L1** was selected for the current study because this ligand set and its derivatives have been utilized in indicator displacement and other sensing schemes under physiological conditions. **L1** was prepared by the condensation of di(2-picolyl)amine and 2,6-bis(chloromethyl)-4-methylphenol in THF in the presence of triethylamine following the reported procedure,[58] and its identity was confirmed by ^{1}H and ^{13}C NMR spectroscopy (Supporting Information). The Zn_2**L1** complex used in complexometric and displacement studies was prepared in situ by the addition of 2 equiv of $ZnCl_2$ to **L1** in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 7.4. Among the various metals that may bind the di(2-picolyl)-amino (DPA) ligating group in **L1,** zinc is the logical choice for a physiological, reversibly binding receptor due to its air stability and the relatively high kinetic lability of its complexes. Furthermore, phenolate/DPA-supported complexes display high affinity (on the nanomolar range in some cases) for Zn^{2+} in living tissue.[59−61] These features suggest that while the indicator may be displaced, Zn_2**L1** itself will not be readily demetalated in biological contexts and is thus a stable scaffold for biosensor applications.

A set of 11 commercially available complexometric indicators were selected for screening (Chart 1). Each indicator shown in Chart 1 was titrated with a solution of preformed Zn_2**L1** complex in HEPES (10 mM, pH 7.4), and absorption changes were monitored by UV−vis spectroscopy. Spectra for one titration (using **ZC**) are presented in Figure 1, while the complete set of titration spectra is provided in the Supporting Information. A modification of the classic Benesi−Hildebrand method[62] was used to extract dissociation constants (K_d) from titration data for each indicator (Table 1; all plots and linear fits are provided in the Supporting Information). Among the indicators screened, K_d values cover 2 orders of magnitude, from 2.8×10^{-4} to 2.7×10^{-6} M (Table 1). Significant variation due to steric and electronic influences is noted even for indicators that presumably bind to Zn_2**L1** through the same ligating unit. For example, indicators binding through a catecholate (**ARS**, **BPR**, **GC**, and **PV**)[47] have K_d values ranging from 2.8×10^{-4} to 2.7×10^{-6} M, and the values for Eriochrome dyes **ERB** and **EBBB**, both of which are expected to bind metals via a phenolate and an azo nitrogen,[63−65] are 4.0×10^{-5} and 8.2×10^{-6} M, respectively. **MX**, which can bind a single metal ion in a tridentate O−N−O fashion,[66] binds within the same range ($K_d = 2.1 \times 10^{-5}$ M). Among the indicators examined, the only K_d previously reported was for the **PV**−Zn_2**L1** complex. The reported value of 1.9×10^{-5} M,[49] determined from calorimetric and UV−vis data in HEPES at pH 7.0 (also reported in TES buffer at pH 7.4),[48] is in good agreement with that determined in the current study (3.0×10^{-5} M). The variation of binding constant with small pH/buffer changes is the likely origin of the difference between the two values. **BPR** and **ZC** bind to Zn_2**L1** most strongly and approximately an order of magnitude more strongly than does **PV**, compared with the weakest binding,

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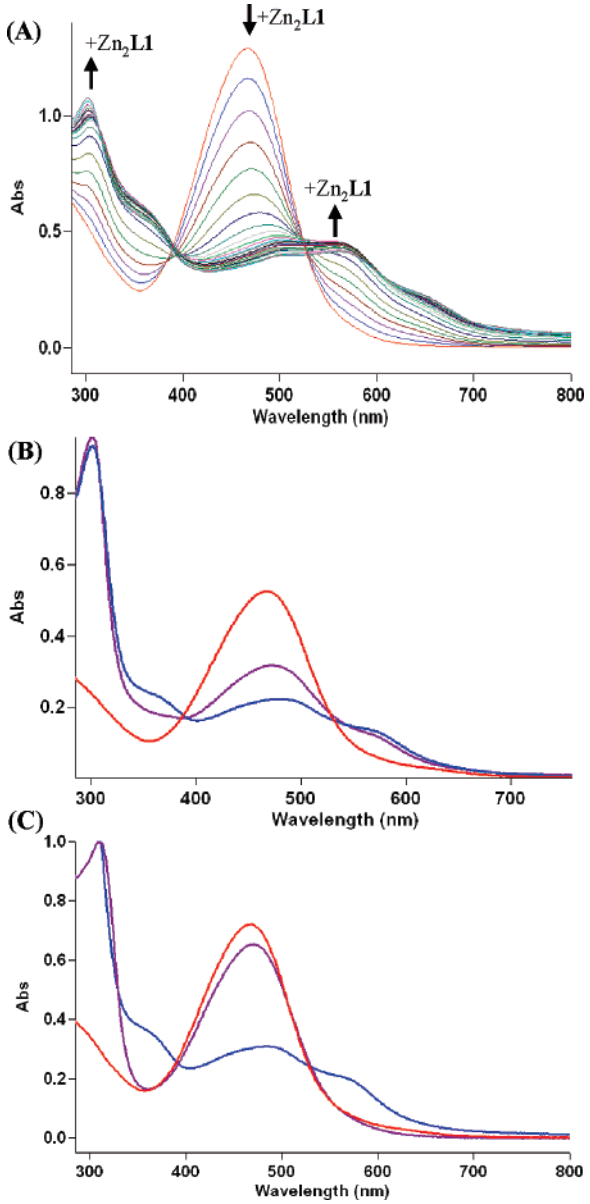

Figure 1. (A) Titration of **ZC** with Zn_2**L1**, followed by UV−vis spectroscopy (the solvent is 10 mM HEPES at pH 7.4). (B) Phosphate displacement test; the red trace corresponds to **ZC**, the blue trace corresponds to the **ZC**−Zn_2**L1** complex, and the purple trace corresponds to the **ZC**−Zn_2**L1** complex in the presence of HPO_4^{2-}. (C) Pyrophosphate displacement test; the red trace corresponds to **ZC**, the blue trace corresponds to the **ZC**−Zn_2**L1** complex, and the purple trace corresponds to the **ZC**-Zn_2**L1** complex in the presence of $H_2P_2O_7^{2-}$.

observed for **ARS**, an order of magnitude less strongly binding than **PV**. The availability of a wide range of K_d values is invaluable for the design of IDAs with specified responses (vide infra). Another parameter of interest in the design of colorimetric IDAs is the extent to which the color changes upon exposure to analyte. Photographs are provided in Table 1 for bound and free forms of the indicators, demonstrating the range of wavelengths available within the indicator series. **EBBB** exhibits the greatest change in λ_{max} between free and Zn_2**L1**-bound states ($\Delta\lambda = 195$ nm, Table 1), making it very easy to follow binding and displacement events by eye. The smallest shift is observed for **MB9**, with $\Delta\lambda_{max}$ of only 12 nm between bound and unbound states.

Table 1. Absorption Data and Photos Demonstrating the Range of Colorimetric Responses Observed in Free, Bound, and Pyrophosphate-Displaced States[c]

Indicator	K_d μM	λ_{free} nm	λ_{bound} nm	$\Delta\lambda$ nm	Displacement[a] HPO_4^{2-}	Displacement[a] $H_2P_2O_7^{2-}$
ARS	280	516	541	25	Y	Y
BPR	2.7	554	578	24	N	Y
DT	13	471	487	16	N	Y[b]
EBBB	8.2	649	454	195	Y	Y
ERB	40	469	510	41	Y	Y
GC	18	621	563	58	Y	Y
MB9	5.1	532	544	12	N	Y
MX	21	520	480	40	Y	Y
PAR	18	412	490	78	Y	Y
PV	30	443	635	192	Y	Y
ZC	2.9	467	563	96	Y	Y

[a] Displacement is considered to take place when there is a >50% return in absorbance at λ_{bound} to the absorbance at that wavelength for the unbound form. [b] Displacement requires ~1 min before it can be observed, whereas all other positive displacement events are observed immediately upon mixing. [c] Absorption spectra for these experiments are provided in the Supporting Information

This change is still visible to the naked eye in a side-by-side comparison but is considerably less pronounced than that for some of the other dyes.

Once the binding of indicators to Zn_2**L1** had been quantitated, phosphate and pyrophosphate were selected as test analytes to gauge their ability to displace indicators with restoration of the "unbound" color. These analytes were selected to allow comparison of the series of indicators screened in this study with previous IDAs for these analytes utilizing the Zn_2**L1** receptor. Furthermore, previous studies have already demonstrated little or no binding affinity of other common anions such as nitrate, sulfate, acetate, or halides to this receptor.[49] Crystallographically characterized compounds of phosphate and pyrophosphate anions with dizinc complexes closely related to Zn_2**L1** indicate their

probable binding modes.[67] Phosphate bridges the two metal centers in a μ-κ^2-*O,O′* fashion.[68] Pyrophosphate binds within the bimetallic cleft of a dizinc complex similar to Zn_2**L1** wherein two oxygens on each P atom coordinate one zinc ion in a κ^2-*O,O′* fashion.[67] Pyrophosphate ($K_d = 1.5 \times 10^{-6}$ M) is bound about 6 times more strongly than phosphate ($K_d = 9.1 \times 10^{-6}$ M) in 1:1 complexes with Zn_2**L1**.[69] On the basis of equilibrium considerations, displacement of >50% of indicator by 1 equiv of analyte will occur when $K_{d,indicator} > K_{d,analyte}$. Assuming that indicator binding is reversible and that there is no significant kinetic barrier to displacement, a colorimetric response corresponding to at least partial restoration of the indicator to its uncomplexed form would be expected in such cases. An indicator that binds to Zn_2**L1** with a K_d value significantly higher than that of both analytes should be easily displaced by either of them, so selectivity cannot be accomplished in these cases. This prediction holds true for six of the seven indicators in this category (**ARS**, **ERB**, **GC**, **MX**, **PAR**, and **PV**), all of which immediate return to the color of the unbound indicator upon the addition of phosphate or pyrophosphate. The exception is **DT**, which requires ~1 min for displacement following anion addition.

Although an IDA for phosphate employing **PV**−Zn_2**L1** was previously noted,[49] pyrophosphate was not tested as a competing analyte, and this IDA cannot distinguish between the two anions at physiological pH. In order to accomplish selective detection of the more strongly binding pyrophosphate from an equal concentration of phosphate, the indicator selected must bind to Zn_2**L1** with a K_d value between the values for phosphate and pyrophosphate (between 1.5 and 9.1 μM). The narrow range afforded by these analytes makes selective detection a challenge. In the current series, **BPR**, **MB9**, and **ZC** have appropriate K_d values expected to facilitate such a selective response.[30] The binding constant for **EBBB** is in this range as well but is nearly identical to that of phosphate, and we were not able to observe selectivity in this case. A selective response to 1 equiv pyrophosphate over equimolar phosphate is evident, both visually and spectroscopically, when **BPR**, **MB9**, or **ZC** is used as the indicator. The selectivity of the **ZC**-based IDA for pyrophosphate (Figure 1c) over phosphate (Figure 1b) is evident from the absorption spectra as well as by eye. The violet **ZC**−Zn_2**L1** complex changes to the orange color typical of free **ZC** immediately upon the addition of pyrophosphate, but changes little upon the addition of phosphate. The phosphate/pyrophosphate displacement described herein is another illustrative example of the IDA strategy's simplicity; a single receptor can be utilized with a range of indicators to give different responses and selectivities that depend on the relative binding affinities of the analyte and indicator selected. The dissociation constants determined in the current study for this set of commercially available indicators should guide their application in future IDAs.

Concluding Remarks

A series of 11 complexometric indicators was investigated for spectral response to a dizinc phosphohydrolase model complex Zn_2**L1**. Dissociation constants determined for 11 of these complexes span 2 orders of magnitude. These 11 indicators were tested for displacement by phosphate and pyrophosphate anions in HEPES buffer at a physiological pH of 7.4. Selective indicator displacement assays for pyrophosphate were discovered using **BPR**, **MB9**, or **ZC** as displaceable indicators. Differentiation of analytes relies heavily on the relative binding constants of the indicator and analyte(s) of interest. The binding data presented herein will guide our future application of biologically applicable sensors utilizing this set of dyes. Work to expand upon these data and to prepare more selective dizinc receptors for anion detection by indicator displacement is currently underway in our laboratory.

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Supporting Information Available: Experimental procedures; proton and ^{13}C NMR spectra for **L1**, UV−vis spectra and Benesi−Hildebrand plots for titrations of Zn_2**L1**, and displacement trials with all 11 indicators. This material is available free of charge via the Internet at http://pubs.acs.org.

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