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Complexation of bisphosphonates with Ytterbium(III): Application of phosphate and ATP detection assay based on Yb³⁺-pyrocatechol violet

Ernestas Gaidamauskas^a, Helen Parker^a, Boris A. Kashemirov^b, Alvin A. Holder^a, Kanokkarn Saejueng^{b,1}, Charles E. McKenna^{b,*}, and Debbie C. Crans^{a,*}

^aDepartment of Chemistry, Colorado State University, Fort Collins, CO 80523-1872

^bDepartment of Chemistry, USC, Los Angeles, CA 90089-0744

Abstract

The coordination chemistry of bisphosphonates with Yb³⁺ was investigated to evaluate the potential of the UV-Vis based detection method using the Yb³⁺-pyrocatechol complexation reaction as a sensor for bisphosphonates. The complexation chemistry of Yb³⁺ with phosphate and ATP analogs was previously described (E. Gaidamauskas et al *J. Biol. Inorg. Chem.* 13 (2008) 1291-1299), and we here study the complexation chemistry of bisphosphonates in this system. The spectrophotometric assay yields direct evidence for formation of a 4:3 metal to ligand complex at neutral pH. Direct evidence for Yb³⁺ : methylenebis(phosphonate) complexes with 1:1 and 1:2 stoichiometry was also obtained by potentiometry at acidic and basic pH. Direct evidence for complex formation was obtained using ¹H NMR spectroscopy although the stoichiometry was not accessed at neutral pH. Our results suggest that the spectroscopic observation of the YbPV complex can be used to conveniently measure concentrations of bisphosphonates down to 2-3 μM .

Keywords

Bisphosphonate coordination; Ytterbium(III); Pyrocatechol violet; Bisphosphonate detection; UV-vis sensor

1. Introduction

Bisphosphonates, a class of compounds used for treatment of osteoporosis and bone cancer and also important in certain industrial processes, form strong coordination complexes with metal ions in solution.[1,2] The formation of highly stable metal bisphosphonate complexes with a reporter group could give rise to a spectroscopic change observable by UV-vis or fluorescence spectroscopies can be used as an analytical method for bisphosphonate detection. This is particularly important for those bisphosphonates that do not contain inherent

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*Corresponding authors: Fax: +1 970 4911801. crans@lamar.colostate.edu (D.C. Crans). Fax: +1 213 740 0930. mckenna@usc.edu (C.E. McKenna)..

¹Current address: Department of Chemistry, Faculty of Science, Khonkaen University, Tumbon Naimaung, Amper Maung, Khonkaen, Thailand 40002

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chromophores. Examples of anti-resorptive bisphosphonate drugs include risedronate, zoledronate, ibandronate, alendronate, pamidronate, etidronate and clodronate, [3-5] of which the latter five lack a well-absorbing UV chromophore. α -Halogenated bisphosphonates are also of current interest as components of nucleotide analogs used to probe enzyme mechanisms [6-9] and as potential anti-tumor agents.[10] The coordination chemistry of solution bisphosphonate species is often similar to pyrophosphates [11,12]. Herein we describe the coordination chemistry of bisphosphonates with Yb^{3+} which, providing the first example of bisphosphonate detection using a ligand displacement reaction.[13]

Coordination chemistry has been previously used as a strategy for development of phosphate [14] and pyrophosphate [15] sensors. A method that has received attention recently is based on the binuclear Zn^{2+} -dipicolylamine based system, which shows exceptionally strong affinity for both organic and inorganic phosphates.[16] Simple mononuclear metal ion complexes have also been reported for phosphate and organic phosphate detection.[13,14] Mononuclear metal ion systems based on displacement assemblies have the advantage of simplicity, but the affinity for phosphate is lower because only one coordination center is involved. In the case of pyrophosphate, this advantage is smaller, because the formation constants for simple single metal-pyrophosphate complexes [17] are similar to the formation constants for dinuclear metal-pyrophosphate complexes.[18-21] In this work, we describe the coordination chemistry of pyrophosphate and several bisphosphonates with a simple Yb^{3+} -center, and demonstrate its potential as a sensor for these ligands.

Prior investigation of the coordination chemistry of bisphosphonates has been limited to speciation studies with simple metal ions.[22-24] Several species form, as is also the case with pyrophosphate, but the most important complex for development of ligand displacement detection methods is a 1:1 metal to ligand complex. The solid material that was isolated from saturated solutions of ytterbium and pyrophosphate was found to have a stoichiometry of 4 to 3,[25] although no other structural data are available for it. The selection of metal ions suitable for sensing purposes must be based on their formation of strong complexes with the bisphosphonate ligand, and thus, the simple speciation chemistry reported points to metals such as Cu^{2+} , Zn^{2+} , and rare earth M^{3+} ions. Direct observation of Cu^{2+} – bisphosphonate complexation in acidic medium has already been exploited for bisphosphonate detection by UV spectrophotometry.[26]

The indirect detection of HPO_4^{2-} and ATP was previously accomplished using a ytterbium (Yb^{3+}) pyrocatechol (PV) complex.[13,27-29] The UV-vis YbPV-method is based on formation of a complex which in the presence of the target ligands undergoes ligand exchange. [13,27-29] The ligand exchange reaction was conveniently monitored at 623 nm by the disappearance of the Yb^{3+} -pyrocatechol complex, with formation of the colorless Yb-P_i (or Yb-ATP) complex. Although there has been a recent study on pyrophosphate sensing,[15] no studies have previously considered the possibility of bisphosphonate detection using ligand displacement sensors.

The chelating properties of bisphosphonates and their several protonation states indicate potential formation of several complexes depending on the pH and the substitution on the bridging methylene between the two phosphorus atoms.[2] As noted above, a 1:1 complex has been structurally characterized for Yb^{3+} and PP_i in the solid state,[30] however, the 4 to 3 solid isolated from aqueous saturated solution [25] has not been structurally characterized. The properties of a range of hydrated rare-earth (including Yb^{3+}) PP_i complexes prepared from hydrothermal synthesis was investigated using XRD, IR, and NMR spectroscopies as well as X-ray crystallography.[31] Solution studies with methylenebis(phosphonic acid) and 1-hydroxyethylidene-1,1-bis(phosphonic acid) suggest the existence of both the 1:1 and 1:2 complexes with other lanthanides.[32] The solid 1:2 complexes between 1-

hydroxyethylidene-1,1-bis(phosphonic acid) and lanthanides yield different stoichiometries when hydrated in solution.[33] Given the number of possible complexes that can form in an aqueous system, any assay based on complexation chemistry must investigate the nature of the complex that gives rise to the spectroscopic signal.

Herein we report studies using the Yb-PV system to define its complexation chemistry in the presence of several bisphosphonates and to evaluate the potential of the Yb-PV system for measuring low bisphosphonate concentrations at neutral pH.

2. Experimental

Reagent Stock solutions

The 10 mM stock solutions of PV and YbCl₃ were prepared by dissolving PV and YbCl₃ 6H₂O (both from Sigma Aldrich) in deionized water. The PV stock solution was standardized at pH 5.3 in 10 mM acetate buffer solution, based on the average molar absorbance value of 14000 M⁻¹cm⁻¹ at 445 nm,[34] and the Yb³⁺ stock solution was standardized by complexometric titration with Na₂H₂EDTA at pH 6.0 in acetate buffer using xylenol orange as an indicator.[35] These studies were developed based on standardization curves similar to those reported previously.[34,35]

Analyte Solutions

The 10 mM analyte stock solutions were prepared by dissolving methylenebis(phosphonic acid), 1-hydroxyethylidenebis(phosphonic acid), anhydrous tetrasodium pyrophosphate (all three from Aldrich), and tetrasodium difluoromethylenediphosphonate [36] in water. The (4-amino-1-hydroxybutylidene)bis(phosphonic acid) monosodium salt trihydrate and [1-hydroxy-2-(3-pyridinyl)ethylidene]bis(phosphonic acid) monosodium salt were donated by Procter & Gamble, and (3-amino-1-hydroxypropylidene)bis(phosphonic acid) was a gift from Novartis. The purity of bisphosphonates was routinely checked by ³¹P NMR and elemental analysis. The analyte stock solution concentrations were verified by potentiometric titration with ~0.2 M KOH under a CO₂-free atmosphere.

General Procedure

The 50 μM YbPV sensor [13] was prepared *in situ* by adding YbCl₃ stock solution first and PV stock solution next to 5.0 mL of 10 mM HEPES buffer at pH 7.0. Aliquots of 10 mM analyte stock solutions were added last with shaking, and after ~1min the UV-vis spectrum was recorded in a quartz cuvette. The UV-Vis spectra were acquired at ambient temperature on a PerkinElmer Lambda 25 spectrophotometer in dual beam mode using 10 mM HEPES buffer as a blank. Water purified by the E-pure system from Barnstead up to 17.8 MΩ·cm was used throughout.

3. Results and Discussion

3.1 Sensing of pyrophosphate and its bisphosphonate analogues by YbPV in 10 mM HEPES buffer at pH 7.0

The complexation of pyrophosphate, and etidronate as a representative bisphosphonate compound, with Yb³⁺ was investigated using the colorimetric assay described by eq. (1).[13, 27-29] The UV-vis spectra of a mixture of 50 μM Yb³⁺ and 50 μM PV in 10 mM HEPES pH 7.0 acquired upon successive addition of pyrophosphate and etidronate are shown in Figure 1.

Absorbance differences at 443 and 623 nm plotted as a function of analyte concentration are shown in Figures 2a and 2b. The Yb^{3+} is sequestered from a relatively weak Yb-PV complex to form a more stable Yb^{3+} -pyrophosphate or Yb^{3+} -etidronate complex. However, in contrast to the linear response to phosphate concentration, showing formation of a 1:1 Yb – phosphate complex as reported previously, the absorbance difference versus analyte concentration plot shows two lines with distinctly different slopes (Figure 2): a linear ΔA response to a low analyte concentration, and a zero-slope (no change in ΔA) response at a high analyte concentration. The intersection point of these two lines at the molar metal to ligand ratio of 1.34 ± 0.03 reflects the 4 : 3 complex stoichiometry. These results suggest that this Yb – analyte complex stoichiometry is different from that of Yb – phosphate, the most simple interpretation corresponding to the empirical formula $\text{Yb}_4(\text{P}_2\text{O}_7)_3$, and $\text{Yb}_4(\text{etidronate})_3$. Identical stoichiometry was observed for methylenebis(phosphonate) and (difluoro)methylenebis(phosphonate) (Figures 2c and 2d), even though the pK_a 's corresponding to the first protonation step in the series of analytes PCF_2P , PP_i , PCH_2P , and etidronate extend from 7.8 (PCF_2P) to 10.7 (etidronate).

The detection limits² for pyrophosphate and etidronate are 1.6 μM and 3.0 μM , respectively. The observed concentration ranges for a linear absorbance response to bisphosphonate addition is from 2 μM to 35 μM . The two phosphonate groups in bisphosphonates are known to act as a “bone hook” in binding to hydroxyapatite [37] and the binding affinity is increased by the availability of an α -OH group.[38] We see no evidence for such an increase in affinity, suggesting that the species in solution and the 4:3 solid state complex do not involve significant coordination by the OH group. Solution studies with Ca^{2+} complexes did not find differences in formation constants based on this OH group either.[39]

3.2 Methylenebisphosphonate interaction with Yb^{3+} as monitored by potentiometric and ^1H NMR titration

Several attempts to probe the Yb^{3+} - PXP_i complex structure at millimolar concentration were made, and the most successful results, unfortunately outside the neutral pH-range, obtained for PCH_2P as a ligand, are presented in this section. Potentiometric studies of Yb^{3+} - pyrophosphate and Yb^{3+} - PCH_2P were attempted in 0.4 M KCl. Insoluble species formation in the reliable pH range from 2 to 11 precluded the potentiometrical characterization of the ytterbium ion solution with pyrophosphate, and only limited pH ranges (2.5-2.7 and 8.5 to 11.2) were accessible in the case of PCH_2P at a metal-to-ligand ratio of 1:4. Only two complex species, $\text{Yb}(\text{PCH}_2\text{P})^-$ and $\text{Yb}(\text{PCH}_2\text{P})_2^{5-}$ were detected by a fitting procedure using PSEQUAD.[40] Attempts to include other species, such as $\text{Yb}_4(\text{PCH}_2\text{P})_3$, were rejected. The soluble species forming in solution detected by potentiometric titration are summarized in Table 1. The formation constants of two complexes $[\text{Yb}(\text{PCH}_2\text{P})]^-$ and $[\text{Yb}(\text{PCH}_2\text{P})_2]^{5-}$ should be viewed with caution due to the very limited pH range available for titration. The titration data analysis shows that for example in 0.5 mM Yb^{3+} and 2 mM $\text{PCH}_2\text{P}^{4-}$ (0.4 M KCl) solution, only the 1:1 complex $[\text{Yb}(\text{PCH}_2\text{P})]^-$ forms at acidic pH (2.5-2.7). After reaching pH~3, precipitation begins, and the amount of precipitate increases upon further addition of a base until neutral pH is attained. The precipitate re-dissolves completely at pH ~8.5. Over the pH range 8.5 to 11.2, both the 1:1 and 1:2 ($[\text{Yb}(\text{PCH}_2\text{P})_2]^{5-}$) complexes form in solution.

²The detection limit was evaluated as the ratio $3 \cdot s/b$ (s is the standard error of the regression, and b is the slope of the linear fit)

A previous study of the Yb - pyrophosphate speciation [25] showed the presence of two soluble species $[\text{Yb}(\text{P}_2\text{O}_7)]^-$, and $[\text{Yb}(\text{P}_2\text{O}_7)_2]^{5-}$, and one insoluble $\text{Yb}_4(\text{P}_2\text{O}_7)_3$ species. Using these formation constants a speciation simulation under our assay conditions (50 μM of Yb^{3+} at pH 7.0) predicts that $\text{Yb}_4(\text{P}_2\text{O}_7)_3$ predominates in the high Yb:PP_i ratio region, whereas after reaching equivalent ratio of Yb:PP_i in the range of 1.33 to 1, the abundance of the 1:1 YbPP complex increases rapidly (Figure S1). We attempted to verify this system using potentiometry, but precipitation in this region prevailed as expected based on the concentrations needed to carry out the experiment.

Under our assay conditions (50 μM Yb^{3+} , 50 μM PP_i, 10 mM Hepes, pH 7.0) we did not observe any precipitates. We provide two possible explanations. First, precipitates may form in solution, but are too fine to be observed visually. Our studies confirmed that at higher (millimolar) concentrations visible precipitation indeed forms. Alternatively, precipitate formation often requires concentrations above the limit defined by the solubility product, and, as a result, precipitates are first formed at higher concentrations. Both types of explanations have merit. The ability to see very low levels of precipitates is limited; we found that at the 50 μM level of material it was difficult to see the precipitate. Alternative interpretations of the results from the spectrophotometric assay include the possibility that the 4:3 complex is a soluble complex. If one assumes formation of a soluble $\text{Yb}_4(\text{P}_2\text{O}_7)_3$ complex, the spectrophotometric data can be explained by invoking the formation of $[\text{Yb}_4(\text{P}_2\text{O}_7)_3]$ with the $\log \beta$ lower limit of 69 ± 1 . We do not favor this interpretation because the potentiometric studies did not support formation of the soluble $\text{Yb}_4(\text{P}_2\text{O}_7)_3$ complex.

^1H NMR studies were also undertaken to obtain more information on this system. Given the paramagnetic nature of Yb^{3+} , the presence of this ion affects the relaxation times of all solution components and line broadening would be anticipated for both free and coordinated PV. In Figure 3 we show the ^1H NMR spectra of solutions of PCH_2P with varying amounts of Yb^{3+} . The presence of Yb^{3+} increases the linewidth as anticipated, but in addition, the chemical shift changes. This is indicative that there is some interaction between the Yb^{3+} and the PCH_2P consistent with complex formation even at very low metal to ligand ratios. As the amount of Yb^{3+} increases, a broad signal at ~ 1.5 ppm is observed and the 2 ppm signal decreases. The 1.5 ppm signal is only observed when the ratio of Yb^{3+} to PCH_2P decreases to 1:4. At a ratio of 1:2, the PCH_2P signal at 1.5 ppm exceeds that of the complex. At the 1:1 ratio this signal is the major signal and very little if any free PCH_2P is detectable. A precipitate was observed at the ratio 1:1 and above (spectra at 2:1 and 4:1 not shown), and we therefore did not attempt to extract stoichiometries for the complex(es).

Two signals are observed in the ^1H NMR spectrum in the presence of PCH_2P . Since one of these is formed at high ligand to metal ion concentration, this signal is due to free ligand or some complex or the equilibrium between the two. Given the high ligand ratio it is likely that some free ligand will exist in solution. Speciation would support the possibility that a complex is formed and if only one complex is formed, that the observed signal is the average of a complexed and free ligand or only the ligand. We favor the possibility that the 2 ppm signal is due to free ligand and not a soluble $\text{Yb}_4(\text{PCH}_2\text{P})_3$ complex. The second broad signal at 1.5 ppm is shifted upfield from the ligand, and only forms at higher metal ion to ligand ratios. This is likely a complex with a stoichiometry near 1:1, which predominates at low concentrations near 1:1.5 metal ion and ligand (and below ratios of 1:1.5).

3.3 YbPV assay response towards the bisphosphonate drugs pamidronate, alendronate and residronate in HEPES buffer at pH 7.0

The possibility that bisphosphonate drugs can be determined from the complex Yb^{3+} forms with PV at pH 7.0 in aqueous solution, was confirmed by the studies described above with etidronate in which the bridge carbon bears a methyl and a hydroxy substituent. The assay was

further used with bisphosphonate drugs in which the methyl group is replaced by a primary aminoethyl, aminopropyl, or 3-pyridylmethyl substituent. The spectra of 50 μM YbPV upon addition of pamidronate, alendronate, and residronate were very similar to those obtained for etidronate. Similar spectroscopic signatures suggest that all these systems form the same types of complexes regardless of the functional groups in the bisphosphonate moiety. In Figure 4 we show the absorbance spectral difference at 443 and 623 nm as a function of pamidronate concentration. As in the case of etidronate, the intersection point at 37 μM of analyte suggests the same 4:3 Yb^{3+} - pamidronate complex stoichiometry. Studies with alendronate and residronate were carried out at neutral pH yielding the same 4:3 stoichiometry for the complexes (data not shown).

Previous approaches to detect phosphates, pyrophosphates and nucleotides have been based on electrochemical,[41] conductive,[42] refractive index [43-45] and fluorescence [46,47] methods and as such are limited by the nature of the analyte or eluent phase. At the price of adding steps to the analytical procedure, chemical derivatization can offer added sensitivity, [47-49] but indirect detection methods also represent a promising strategy for analytical detection of these classes of compounds.[42,50-53] Existing spectrophotometric methods for specific bisphosphonates that lack an inherent chromophore have been based upon ceric sulfate oxidation [54,55] (etidronate) or ninhydrin [55] (alendronate) show similar sensitivity, with detection limits from 3 μM to 6 μM . More sensitive spectrophotometric assays for risedronate based on a Cu^{2+} complex have been reported in the literature [56] with a claimed detection limit of 95 nM. This method exploits the intrinsic high molar absorptivity of the pyridine side chain substituent of the drug. Our new indicator displacement assay using the YbPV complex as a sensor is general and including bisphosphonates lacking any chromophore. This approach has the further advantage of being equivalently sensitive for all tested bisphosphonate compounds. It is likely that this sensor can be usefully applied to detecting other bisphosphonates with featureless UV-vis spectra, such as clodronate, ibandronate and others.

In our previous study [13] we showed that YbPV is sensitive to phosphate and ATP, thus one limitation of this new bisphosphonate sensor is that it will not be selective relative to these species, which if present would need to be removed before analysis. Work is in progress to find more selective sensing systems.

4. Conclusion

The coordination complexes that form between Yb^{3+} and bisphosphonates were investigated at pH 7.0 to explore the feasibility of using this chemistry to measure bisphosphonate concentrations by visible spectroscopic detection of PV displaced by the bisphosphonate from an initial YbPV complex. Previous literature relating to the pyrophosphate ligand suggested that the complex should form either a 1:1, a 1:2 or a 4:3 solid.[25] We found direct evidence by complex formation by potentiometry, UV-vis and ^1H NMR spectroscopy. Evidence for both 1:1 and 1:2 complexes Yb-methylenebis(phosphonate) complexes by potentiometry at acidic and basic pH values was obtained, and for a 4:3 complex at neutral pH by a novel spectrophotometric assay we recently described.[13]

Although the Yb^{3+} complex that forms with pyrophosphate is somewhat stronger than that with the bisphosphonates examined as potential ligands, the bisphosphonate complexes were sufficiently strong to quantitatively displace PV from YbPV. Equivalent results were obtained with bisphosphonate drugs having alkyl, aminoalkyl or heterocyclic side chains (etidronate, pamidronate, alendronate and risedronate). This assay will measure total phosphorus content in samples containing multiple phosphorus compounds because of the high affinity for a range of phosphates. The advantage of this simple, sensitive (low μM detection limit) colorimetric sensor for direct spectrophotometric detection of bisphosphonates resides in the fact that the

displaced indicator spectrum is independent of the analyte and thus should be applicable to many bisphosphonate compounds with diverse bridge carbon substitution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PCF₂P, (difluoro)methylenebis(phosphonate); PCH₂P, methylenebis(phosphonate); PXP, generic formula for a bisphosphonate with a X group between the two phosphate groups; P_i, phosphate; PP_i, pyrophosphate; PV, pyrocatechol violet; NMR, nuclear magnetic resonance; Yb-PV, this means a complex formed between Yb³⁺ and PV.; Yb³⁺, ytterbium +3 cation.

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Figure 1.

Absorbance spectra of 50 μM of Pyrocatechol Violet (PV), 50 μM of Yb^{3+} -PV complex (YbPV), and 50 μM of YbPV upon successive addition of tetrasodium pyrophosphate PP_i (a) and etidronate (b) in 10 mM HEPES buffer (pH 7.0)

Figure 2.

Absorbance differences in 50 μM Yb^{3+} and 50 μM PV solutions in 10 mM HEPES buffer (pH 7.0) as a function of added pyrophosphate (a), etidronate (b), methylenebis(phosphonate) (c), and (difluoro)methylenebis(phosphonate) (d) concentrations at two wavelengths (443 and 623 nm). Error bars shown in figures (a) and (b) are the standard deviations of three replicates.

Figure 3.

^1H NMR spectra of 2 mM PCH_2P solutions with increasing amounts of Yb^{3+} . The Yb^{3+} to PCH_2P ratio is shown next to each spectrum. The solution pH's are between 7.0 and 7.2. Precipitation was observed in samples of ratios 1:1 and 1:2. An * denotes an additional broad resonance appearing upon addition of Yb^{3+} .

Figure 4. Absorbance difference in the 50 μM Yb^{3+} and 50 μM PV solution in 10 mM HEPES buffer (pH 7.0) as a function of pamidronate concentration.

Table 1

Species formation constants (as logarithms of total formation constants) in the ternary system H^+ - PXP^{4-} - Yb^{3+} in 0.4 M KCl at 25°C.

Species	$\text{H}(\text{PCH}_2\text{P})$	$\text{H}_2(\text{PCH}_2\text{P})$	$[\text{Yb}(\text{PCH}_2\text{P})]^-$	$[\text{Yb}(\text{PCH}_2\text{P})_2]^{5-}$
$\log \beta$	$9.90 \pm 0.02^*$	$16.61 \pm 0.04^*$	14.1 ± 0.4	18.9 ± 0.4

* The two pK_a 's of methylenebis(phosphonic acid) are equal to 9.90 (± 0.02) and 6.71 (± 0.06).