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Phosphodiester cleavage by trivalent lanthanides in the presence of native cyclodextrins

Mariel Ruiz Kubli, Anatoly K. Yatsimirsky*

Facultad de Química, Universidad Nacional Autónoma de México, México D.F. 04510, Mexico

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ABSTRACT

Testing phosphodiesterase activity of Eu(III) in the presence of native cyclodextrins revealed capacity of β -cyclodextrin (β -CD) to stabilize catalytically active metal hydroxocomplexes in mildly basic solutions. Kinetics of the hydrolysis of bis(4-nitrophenyl) phosphate (BNPP) and transesterification of 2-hydroxypropyl 4-nitrophenyl phosphate (HPNP) as models of DNA and RNA respectively has been studied with La(III), Pr(III), Nd(III), Eu(III), Gd(III) and Dy(III) cations in the presence of β -CD in the range of pH 7.0–9.0. The overall catalytic effect with 2 mM lanthanide– β -CD complexes was up to 10⁵ for HPNP and 10⁸ for BNPP at pH 8 demonstrating the highest catalytic activity among so far reported artificial phosphodiesterases. Analysis of concentration and pH-dependences of observed rate constants for different lanthanides showed that active species are binuclear polyhydroxocomplexes of general type [M₂(β -CD) (OH)_n]⁶⁻ⁿ with n = 3-5. The metal– β -CD and phosphodiester– β -CD interactions were studied by ¹H NMR spectroscopy. Mechanistic implications of much higher catalytic efficiency in BNPP hydrolysis as compared to HPNP transesterification are discussed.

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1. Introduction

Native cyclodextrins form complexes in water with metal ions of different types including lanthanides [1]. Ability of cyclodextrins to stabilize trivalent lanthanide ions against precipitation in basic media was demonstrated long time ago by Komiyama and co-workers [2]. The authors proposed formation of both 1:1 and 2:1 metal cyclodextrin (CD) complexes on basis of solubility data, demonstrated catalytic activity of the complexes in the hydrolysis of a phosphate monoester but did not determine the stability constants and did not extend the reactivity studies beyond testing of just one phosphate monoester as a substrate. Fatin-Rouge and Bünzli [3] reported a detailed study of lanthanide complexation by different CDs both in acid and basic solutions. Stability constants for aqua-ions were in the range $10^2\text{--}10^4\,M^{-1}$ and did not vary significantly for α -, β - and γ -CD. Spectroscopic data confirmed inclusion of cations into CDs, which behaved like crown ethers with anomeric oxygen atoms being possible binding sites. In strongly basic solutions at pH > 12 complexation with deprotonated secondary OH groups was the predominant form of binding. In a more recent study lanthanides were found to form equally

* Corresponding author. E-mail address: anatoli@unam.mx (A.K. Yatsimirsky). stable complexes with anhydrocyclodextrins lacking OH groups [4], which are principal binding groups for transition metal ions [1]. This confirms the original idea [3] that cyclodextrins behave towards lanthanides essentially as crown ethers affording inclusion complexes via ion-dipole contacts with neutral oxygen atoms.

Hydroxocomplexes of trivalent lanthanides constitute an important group of most efficient catalysts for the hydrolysis of phosphate esters including DNA, RNA and model compounds [5]. Development of these catalysts depends critically on the use of appropriate stabilizing ligands, which must prevent precipitation of metal hydroxide and at the same time allow for formation of active hydroxocomplexes. Results of cited above studies point to a possibility that CDs may act as such ligands. Metal-free cyclodextrins [6] as well as metal complexes of chemically modified cyclodextrins [7] were studied as artificial phosphodiesterases, but the potential of lanthanides stabilized by native CDs was not explored yet. Previously we reported successful catalysis by lanthanides stabilized by neutral polyol ligands like bis-Tris propane or Tris [8,9]. Like CDs these compounds act as neutral O-donor ligands using non-dissociated hydroxyl groups as metal binding sites [9,10]. They form rather weak complexes with lanthanide aqua-ions with stability constants in the same range as with CDs, but stabilize active binuclear hydroxocomplexes providing much higher phosphoesterolytic activity than that observed with free metal ions.







In this paper we demonstrate that similar effect is observed with CDs, which act as somewhat less powerful stabilizing ligands, but induce formation of significantly more active lanthanide species at lower pH. The catalytic activity was studied with two model phosphate diesters: bis(4-nitrophenyl) phosphate (BNPP), which is considered as a model for DNA hydrolysis, and 2-hydroxypropyl 4-nitrophenyl phosphate (HPNP), which is considered as a model for RNA transesterification. all β -CD signals indicating stronger although less specific interaction with hydroxo complexes of Nd(III). These observations confirm ability of β -CD to interact with potentially catalytically active hydroxo complexes of lanthanides [5], which explains both the stabilizing effect of β -CD and observed catalytic activity.

Potentiometric titrations of lanthanide salts (Eu(III), Nd(III)) and β -CD were reproducible only at pH below 8 where the fraction of hydroxo complexes was still very low and species assignment



Comparison of results of this study with previously reported results for other lanthanide-based artificial phosphodiesterases shows that CD-stabilized complexes in mildly basic solution at pH 8 reach the level of catalytic activity typical for the most active systems and in some cases even surpass their activity making lanthanide/ β -CD system a simple practically useful artificial phosphodiesterase catalyst.

2. Results and discussion

In preliminary experiments we observed that the catalytic activity of lanthanides is significantly higher with β -CD than with α - or γ -CD (Fig. S1, Supplementary Material). All studies therefore were performed with β -CD. In the concentration range of lanthanides from 0.5 to 2.5 mM addition of 1 equivalent of β -CD was sufficient to prevent precipitation of metal hydroxide on increase in pH approximately up to pK_a value of the respective cation, e.g. up to pH 9 with less acidic La(III) and up to pH 8 with most acidic Dy(III). In the absence of CD precipitation with 1 mM lanthanides starts already at pH 7. An excess of CD did not improve the stability. To control the reaction pH the 50 mM Tris/HCl buffer was employed. The buffer by itself has some stabilizing effect but the rate constants measured in the absence of β -CD were at least one order of magnitude smaller than those in the presence of β -CD.

Attempts to characterize the interactions of lanthanides with β -CD in more details as compared to those reported by Fatin-Rouge and Bünzli [3], in particular establish the type of hydroxo complexes, provided limited information. Lanthanides affect very little the ¹H NMR spectra of β -CD in acid solutions. Fig. 1 shows the ¹H NMR spectra of β -CD alone and in the presence of some lanthanide cations at pH 5.5. A single noticeable metal-induced change is a down-field shift of the signal of H-5 proton located in the interior of the CD cavity [11]. The shift is very small for La(III), but larger for more acidic Eu(III) and Nd(III) cations for which the signal overlaps with more intense signal of H-6 protons and with increased concentration of Nd(III) it is shifted further to a more down-field position (upper spectre in Fig. 1). These results confirm inclusion of lanthanide cations into β -CD cavity.

More importantly the interaction between a lanthanide cation and β -CD becomes stronger at higher pH values. Fig. 2 shows ¹H NMR spectra of β -CD in the presence of Nd(III) recorded at increased pH, which demonstrates that at pH 7 besides the signal of H-5 the cation shifts also the position of H-3, another interior proton of β -CD, and at pH 8 one observes a strong broadening of was very uncertain. Therefore a possible composition of active species was inferred from kinetic results obtained at variable pH.

Besides interactions with metal ions β -CD may form inclusion complexes with substrates possessing hydrophobic nitrophenyl groups, which also may affect the observed reactivity. A complexation of diphenyl phosphate with β -CD with the binding constant $K = 200 \text{ M}^{-1}$ determined by fluorescence titration was reported previously [12]. Fig. 3(A) shows the course of ¹H NMR titration of β -CD by BNPP in D₂O. No shifts of the signals of external H-2 and H-4 protons is observed, but the signals of internal H-3 and H-5 protons undergo strong up-field shifts consistent with inclusion of aromatic groups of BNPP inside the CD cavity. The complexation induced shifts in the signals of H-5 and H-3, which sit at opposite sides of the cyclodextrin cavity, are similar by their magnitude which means that BNPP can enter the cyclodextrin cavity from both sides. The profile of the signal of H-3 proton vs. guest concentration is shown in Fig. 3(B) and the fitting of this profile to a 1:1 binding isotherm gives the binding constant $K = 320 \pm 20 \text{ M}^{-1}$ for BNPP. Similar experiment with HPNP showed much smaller complexation-induced shifts in the signals and a linear profile (Fig. 3 (B)), which does not allow one to estimate a significantly smaller binding constant in this case.

Curiously, recent theoretical calculations predict external BNPP binding to β -CD via H-bonding of the host OH groups to the phosphoryl group of the phosphodiester, [13] which obviously contradicts the experimental results.

On basis of determined binding constant for BNPP one may conclude that with β -CD concentrations below 2.5 mM employed in kinetic studies (see below) the degree of the substrate complexation by CD is less than 50% and for HPNP it should be even smaller.

In all kinetic experiments hydrolysis of BNPP proceeded though intermediate formation of mono-4-nitrophenyl phosphate, which further was hydrolyzed to the second 4-nitrophenolate anion and inorganic phosphate but with a different smaller rate constant. The details of kinetic analysis are given in the Section 4. The observed first-order rate constants (k_{obs}) for BNPP hydrolysis discussed below correspond to the first step of the reaction. In the case of HPNP usually overlooked problem is that the compound prepared by traditionally employed procedure [14] contains ca. 5% of 1-hydroxy-2-propyl isomer, which is ca. 10 times more reactive than the main 2-hydroxypropyl isomer [15] (see also [16]). The contribution from the hydrolysis of the more reactive isomer practically disappears after ca. 20% of hydrolysis and this initial part of the reaction was excluded from the calculation of the rate constant.



Fig. 1. The ¹H NMR spectra of 5 mM β-CD alone and in the presence of some lanthanide cations at pH 5.5 (uncorrected pH value in D₂O, 0.05 M MES buffer, the range between 3.64 and 3.78 ppm containing the buffer signals is eliminated).



Fig. 2. ¹H NMR spectra of 5 mM β-CD alone and in the presence of 4 mM Nd(III) at increased pH (0.05 M TRIS/HCl buffer, the range between 3.66 and 3.84 ppm containing the buffer signal is eliminated; uncorrected pH values in D₂O are indicated).

Fig. 4A shows the profile of k_{obs} vs. concentration of the equimolar mixture of Eu(III) and β -CD at constant pH 8.0. Evidently at low concentrations the profile is curved upward, but above 1 mM it becomes linear. The same results in logarithmic coordinates (Fig. 4B) demonstrate that initially the reaction rate is a quadratic function of the concentration and above 1 mM it becomes a linear function. Similar profile was obtained with HPNP as a substrate (Fig. 2S, Supplementary Material). Such behavior agrees with a self-assembling process of catalytically inactive mononuclear metal–CD complexes into active binuclear complexes above 1 mM total metal concentration. In the presence of 2 mM Eu(III) the reaction rate was similar with 1 or 2 mM β -CD, but with 2 mM β -CD reaction rate was proportional to metal ion concentration above 1 mM. Such behavior agrees with formation of catalytically active complexes of the composition $M_2(\beta$ -CD). All further kinetic experiments were performed with



Fig. 3. (A) ¹H NMR titration of 2 mM β-CD by BNPP in D₂O. (B) Chemical shift of H-3 proton vs. concentration of BNPP or HPNP in D₂O.



Fig. 4. (A) Dependence of the observed first-order rate constant for the BNPP hydrolysis at pH 8.0 on the concentration of Eu(III) in the presence of 1 equivalent of β -CD. (B) The same results in the logarithmic coordinates. Lines are drawn to visualize the trend and are not theoretical fits.

2 mM total metal when the reaction was first-order in the catalyst and although the presence of half of equivalent of β -CD was sufficient to observe the maximum catalytic activity, 2 mM β -CD was employed because of better stability and reproducibility with 1:1 metal:CD ratio.

Lanthanide cations are very prone to self-association through hydroxide bridges [17]. In a recent detailed study of speciation of Eu(III) by direct excitation luminescent spectroscopy formation of binuclear $[Eu_2(\mu-OH)_2(H_2O)_{16}]^{4+}$ species was detected together with aquo-ion $[Eu(H_2O)_9]^{3+}$ and mono-hydroxide complex [Eu $(OH)(H_2O)_8]^{2+}$ even at pH 6.5 in a micromolar concentration range [18]. One may expect therefore that the concentration profile in Fig. 1 reflects transformation of mononuclear to binuclear hydroxide bridged complexes of general type $[Eu_2(\mu-OH)_2(\beta-CD)(OH)_n]^{4-n}$. Previous results for lanthanides stabilized by polyol ligands [8,9] indicate that the bridging hydroxide ions lack the nucleophilic reactivity and for this reason the active species must have an additional number of non-bridging hydroxides, the number of

which will be estimated on basis of pH-profiles of reaction rates discussed below.

Figs. 5 and 6 show the pH-profiles of the rates of catalytic hydrolysis of BNPP and HPNP respectively at fixed 2 mM concentration of different lanthanides in the presence of 2 mM β -CD.

With both substrates the reaction rate shows a very strong dependence on pH. Plots of log k_{obs} vs. pH are linear until pH is approaching pK_a of the respective cation (9.33; 8.82; 8.70; 8.59; 8.62; 8.37 for La(III), Pr(III), Nd(III), Eu(III), Gd(III), Dy(III) respectively [19]), but at higher pH values solutions become unstable and results are poorly reproducible. The slopes of the plots roughly indicate the number of hydroxide anions bound to the metal to form a reactive species. Alternatively they may reflect the number of deprotonated OH groups of β -CD which may act as nucleophiles towards BNPP in form of alkoxide anions [6]. In this case the phosphorylated β -CD should be the reaction product. To explore this possibility the reaction was monitored by ³¹P NMR in the presence of 2 mM β -CD, 2 mM La(III) and 2 mM BNPP at pH 9.0 until nearly



Fig. 5. Observed first-order rate constants (s⁻¹) for the hydrolysis of BNPP in the presence of 2 mM β -CD and 2 mM metal ion vs. pH.



Fig. 6. Observed first-order rate constants (s⁻¹) for the hydrolysis of HPNP in the presence of 2 mM β -CD and 2 mM metal ion vs. pH.

complete release of 4-nitrophenol measured spectrophotometrically. The phosphorylated β -CD shows a characteristic signal at 3.2 ppm [6], but only signals of BNPP (-11.28 ppm), mono(4-nitrophenyl) phosphate (-0.58 ppm) and inorganic phosphate (2.41 ppm) were observed during the reaction course. No phosphorylated β -CD was detected also with HPNP as a substrate.

With BNPP as a substrate the slopes are similar for all cations besides Dy(III). The slope for Dy(III) is 2.70 ± 0.05 and for all other cations it equals 2.1 ± 0.1 . With HPNP as a substrate the reaction rate in the presence of La(III), Pr(III) and Nd(III) initially is proportional to free hydroxide concentration (slope 1 in the logarithmic coordinates), but then the reaction rate sharply increases and log k_{obs} becomes a linear function of pH with the slope 2.5 ± 0.2 for all three cations. For reactions in the presence of Eu(III), Gd(III) and Dy(III) only the slope 2.5 ± 0.2 is observed (Fig. 6).

Linear plots in Figs. 5 and 6 without "saturation" indicate a small degree of formation of hydroxo complexes when their concentrations are still proportional to the concentration of free

Table 1

Observed first-order rate constants for the cleavage of phosphodiesters by 2 mM lanthanide cations in the presence of 2 mM β -CD at pH 8.0.

	BNPP		HPNP	
	$k_{ m obs}$, s $^{-1}$	$k_{\rm obs}/k_0^{\rm a}$	$k_{ m obs}$, s ⁻¹	$k_{\rm obs}/k_0^{\rm a}$
La(III) Pr(III) Nd(III) Eu(III) Gd(III) Dy(III)	$\begin{array}{c} 1.2\times 10^{-5}\\ 6.0\times 10^{-5}\\ 1.2\times 10^{-4}\\ 6.0\times 10^{-4}\\ 1.0\times 10^{-3}\\ 8.0\times 10^{-4}\end{array}$	$\begin{array}{c} 1.2\times 10^{6}\\ 6.0\times 10^{6}\\ 1.2\times 10^{7}\\ 6.0\times 10^{7}\\ 1.0\times 10^{8}\\ 8.0\times 10^{7}\end{array}$	$\begin{array}{c} 7.0 \times 10^{-4} \\ 9.0 \times 10^{-4} \\ 2.3 \times 10^{-3} \\ 1.0 \times 10^{-2} \\ 3.0 \times 10^{-2} \\ 4.0 \times 10^{-2} \end{array}$	$\begin{array}{c} 4.1 \times 10^{3} \\ 5.3 \times 10^{3} \\ 1.3 \times 10^{4} \\ 6.0 \times 10^{4} \\ 1.8 \times 10^{5} \\ 2.3 \times 10^{5} \end{array}$

^a Rate acceleration over the rate of spontaneous hydrolysis at pH 8.0; $k_0 = 10^{-11}$ s⁻¹ for BNPP and 1.7×10^{-7} s⁻¹ for HPNP [5b].

hydroxide in power "*n*" where "*n*" is a number of hydroxide anions bound to a catalytically active form of the complex. Assuming that at lower pH with 2 mM metal and 2 mM β -CD the predominant species are binuclear hydroxide bridged complexes of general composition $[M_2(\mu$ -OH)₂(β -CD)]⁴⁺ the respective equilibrium can be expressed as the Eq. (1) with "*n*" ranging from 1 to 3.

$$\left[\mathsf{M}_{2}(\mu\text{-}\mathsf{OH})_{2}(\beta\text{-}\mathsf{CD})\right]^{4+} + n\mathsf{OH}^{-} \rightleftharpoons \left[\mathsf{M}_{2}(\mu\text{-}\mathsf{OH})_{2}(\beta\text{-}\mathsf{CD})(\mathsf{OH})_{n}\right]^{4-n} \quad (1)$$

The fractional values of "*n*" like e.g. 2.5 can be explained by similar contributions of complexes with n = 2 and 3 to the reaction rate. Such behavior agrees very well with previous results for phosphoesterolytic activity of lanthanides stabilized by polyol [8,9] and amino acid ligands [20] where the binuclear hydroxo complexes of similar type with "*n*" ranging from 1 to 3 were proved to be the active species.

To illustrate the catalytic efficiency of cyclodextrin-stabilized complexes Table 1 collects the observed rate constants determined at a single pH 8.0 for different 2 mM metal ions. Let us consider first the results for BNPP. The catalytic effect over the rate of spontaneous hydrolysis at pH 8 ($k_0 = 1 \times 10^{-11} \text{ s}^{-1}$ [21]) reaches the factor of 10⁸ (Table 1) which is among the largest for all reported so far in aqueous solutions (larger acceleration factors were observed in methanol [22] and 80% vol. aqueous DMSO [23]: accelerations of the order $10^8 - 10^9$ in water were reported for Ce(IV) ions [24] with BNPP, Zr(IV) complexes with dimethyl phosphate [25] and Lu(III) with diribonucleotides [26]). Among lanthanide complexes of bis-Tris propane at similar pH and metal ion concentration the most active is Pr(III) with $k_{obs} = 8.0 \times 10^{-5} \text{ s}^{-1}$ [9] while the cyclodextrin Gd(III) complex shows a one order of magnitude higher activity. The k_{obs} values up to $2.5 \times 10^{-3} \text{ s}^{-1}$ were observed for lanthanide complexes of 2(4)-imidazole carboxylic acid [20a], which are the most active lanthanide based catalysts for BNPP hydrolysis, but at pH above 9. As one can see from Fig. 5 similar activity can be reached with β -CD Nd(III) complex at pH below 9. The general trend of increased activity for heavier and more acidic lanthanides reflects larger degree of formation of hydroxo complexes at chosen pH below pK_a values for all cations.

Considering results for HPNP we observe the catalytic activity also similar to that reported for complexes of 2(4)-imidazole carboxylic acid [20a] and even a little bit higher than that for a binuclear macrocyclic Eu(III) complex ($k_{obs} = 2.5 \times 10^{-2} \text{ s}^{-1}$; estimated from results of [27] at 2 mM Eu(III) and pH 8.0), which is the best lanthanide-based catalyst for this substrate. We observe similar general trend in activity of different lanthanide cations increasing from La(III) to Dy(III), but much lower catalytic effect over the rate of spontaneous hydrolysis of HPNP as compared with effect in BNPP hydrolysis (Table 1, last column). By itself HPNP is ca. 10⁴ times more reactive than BNPP (see footnote to Table 1), but rate constants of catalytic hydrolysis are on average only 10 times higher for HPNP for than for BNPP. Previously similar effect was noticed for lanthanide complexes of 2(4)-imidazole carboxylic



Scheme 1. $(R = C_6H_4NO_2-p)$.



Scheme 2. Mechanisms of catalysis for the cleavage of a DNA model phosphodiester (A) and an RNA model phosphodiester (B).

acid [20a]. Also the efficiency of HPNP cleavage is much smaller than that of BNPP with Zn(II) complexes of ligands derived from bis-2-pyridinylmethylamine, which bear additional hydroxyl or aromatic amino groups [28], but efficiency of catalysis by Zn(II) complexes of a series of simple tridentate polyamino ligands is approximately similar for both substrates [29].

The generally more efficient catalysis in BNPP reaction can be explained considering the difference in mechanisms of BNPP and HPNP hydrolyses. The hydrolysis of BNPP proceeds as a direct nucleophilic attack of hydroxide anion on the phosphoryl group [30], but the cleavage of HPNP is a transesterification reaction, which both as a spontaneous [31] and as a catalytic process [32] involves equilibrium predissociation of hydroxyl group with subsequent nucleophilic attack of the alkoxide anion on the phosphodiester group (Scheme 1a,b).

This means that the coordinated hydroxide ion of the catalytically active hydroxocomplex may act as a nucleophile towards bound BNPP (Scheme 2a) with increased reactivity due to two factors: the proximity effect since OH⁻ now acts intramolecularly [33] and the electrophilic assistance of the metal ion to the phosphoryl group. In the case of HPNP the actual active form of the catalyst is the respective aqua complex [32], the nucleophile already is intramolecular and the catalytic effect is only due to electrophilic assistance of the metal ion to the phosphoryl group (Scheme 2b). Evidently the overall acceleration effect should be lower in the case of HPNP and a most viable way to improve the catalysis towards this substrate as well as to RNA is to provide additional electrophilic assistance to the nucleophilic attack on the phosphoryl group [34].

The type of catalytically active hydroxocomplexes of lanthanides stabilized by β -CD namely $M_2(OH)_n^{6-n}$ is the same as that with simple polyol or amino acid ligands, but the reactivity is considerably higher. This can be attributed to ability of β -CD to form at least weak inclusion complexes with the substrates affording ternary metal ion-CD-phosphodiester complexes. Inclusion of a phenyl group into β -CD cavity leaves sufficient free space inside the cavity and formation of ternary complexes of β -CDs with aromatic guests and e.g. alcohols is well documented [35]. Formation of a ternary complex will convert a bimolecular catalytic reaction into a much faster intramolecular process the relative efficiency of which is usually expressed in terms of Effective Molarity (EM) [36]. The EM values for inclusion complexes generally are not high [37], but even a modest EM close to unity will provide a 1000-fold rate acceleration with reactants in mM concentration range employed in this study.

3. Conclusions

In conclusion, using β -CD as a stabilizing ligand for hydroxo complexes of trivalent lanthanides provides a very simple means of obtaining highly active phosphodiesterase catalysts operating in mildly basic nearly neutral aqueous solutions. In the presence of 2 mM Gd(III)- β -CD complex at pH 8 the half-life of BNPP is reduced from 2000 years to 10 min and that of HPNP from 48 days to just 20 s. The rate-concentration and the rate-pH profiles indicate the binuclear polyhydroxo structure of the active complexes. The system suffers to some extent from the difficulty to obtain its full characterization, but it is compensated by simplicity of generating highly active catalytic species.

4. Experimental

4.1. Materials

2-Hydroxypropyl 4-nitophenyl phosphate (HPNP) was prepared as the barium salt according to the literature procedure [14]. All other reactants were commercial samples obtained from Sigma– Aldrich. Bis(4-nitrophenyl) phosphate (BNPP) was recrystallized from ethanol–water. Cyclodextrins, Trizma base and reagent-grade lanthanide(III) perchlorates as 40 wt% aqueous solutions were used as supplied. Distilled and deionized water (Barnstead Nanopure system) was used for preparation of all solutions.

4.2. NMR spectroscopy

 1 H NMR spectra were recorded on a Varian Gemini 200 NMR spectrometer in D₂O in TRIS or MES 50 mM buffers at appropriate pH.

4.3. Kinetics

The course of BNPP hydrolysis and transesterification of HPNP was monitored spectrophotometrically by the appearance of 4-nitrophenolate anion at 404 nm. Kinetic measurements were performed on a Hewlett–Packard 8453 diode array spectrophotometer equipped with a thermostatted cell compartment at 25.0 ± 0.1 °C. Reaction solutions were prepared by combining appropriate amounts of the metal salt in 50 mM Tris/HCl buffer and β -CD. Reactions were initiated by adding an aliquot of the substrate stock solution to final concentration 50–100 μ M. The observed first-order rate constants (k_{obs}) were calculated by the integral method or, for slow reactions, from initial rates.

With HPNP as a substrate the initial portion of the kinetic curve corresponding to 20–30% of hydrolysis was excluded to avoid interference with more reactive isomer [15,16]. With BNPP as a

substrate more complex two-exponential kinetics was observed and fitted to the Eq. (2).

$$A = \varepsilon_{\rm NP}[{\rm BNPP}]_0 \{ 2 + (k_1 e^{-k2t} + (k_1 - 2k_2)e^{-k1t})/(k_2 - k_1) \}$$
(2)

where *A* is the absorbance, ε_{NP} and [BNPP]₀ are the molar absorptivity of *p*-nitrophenolate and initial substrate concentration respectively, k_1 and k_2 are the first and the second steps of BNPP hydrolysis to the monoester and inorganic phosphate respectively [9]. Given in the text k_{obs} values correspond to k_1 .

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ica.2015.10.039.

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