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Metal-Catalyzed Phosphodiester Cleavage: Secondary ^{18}O Isotope Effects as an Indicator of Mechanism

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Abstract: Information about the transition states of metal-catalyzed hydrolysis reactions of model phosphate compounds has been obtained through determination of isotope effects (IEs) on the hydrolysis reactions. Metal complexation has been found to significantly alter the transition state of the reaction from the alkaline hydrolysis reaction, and the transition state is quite dependent on the particular metal ion used. For the diester, ethyl *p*-nitrophenyl phosphate, the nonbridge ^{18}O effect for the hydrolysis reactions catalyzed by Co(III) 1,5,9-triazacyclononane and Eu(III) were 1.0006 and 1.0016, respectively, indicative of a slightly associative transition state and little net change in bonding to the nonbridge oxygen. The reaction catalyzed by Zn(II) 1,4,7,10-tetraazacyclododecane had an ^{18}O nonbridge IE of 1.0108, showing the reaction differs significantly from the reaction of the noncomplexed diester and resembles the reactions of triesters. Reaction with Co(III) 1,4,7,10-tetraazacyclododecane showed an inverse effect of 0.9948 reflecting the effects of bonding of the diester to the Co(III). Lanthanide-catalyzed hydrolysis has been observed to have unusually large ^{15}N effects. To further investigate this effect, the ^{15}N effect on the reaction catalyzed by Ce(IV) bis-Tris propane solutions at pH 8 was determined to be 1.0012. The ^{15}N effects were also measured for the reaction of the monoester *p*-nitrophenyl phosphate by Ce(IV) bis-Tris propane (1.0014) and Eu(III) bis-Tris propane (1.0012). These smaller effects at pH 8 indicate that a smaller negative charge develops on the nitrogen during the hydrolysis reaction.

Introduction

Phosphodiesters are well-known for both their importance as biomolecules and their stability, with some phosphodiesters having exceptional stability. For instance, the half-life for hydrolysis of double-stranded DNA is estimated to be well over 100 000 years.[1] The stability of phosphodiesters makes them excellent systems for information storage, but at the same time, there must be enzymes that hydrolyze phosphodiester bonds when needed. It is often found that the enzymes that are involved in bond cleavage contain metal ions. For instance, P1 nuclease[2] contains a trimeric zinc center, and other nucleases contain Mg(II) or Fe(III) as well as Zn(II).[3]

In order to understand the enormous rate accelerations of phosphodiester hydrolysis provided by the nucleases, a number of metal-containing model systems have been synthesized that attempt to mimic the enzymes in reactions with model diesters.[4] The effectiveness of these model systems generally results from the intramolecular nucleophile generated by the metal ion at physiological pH as well as Lewis acid activation of the substrate. The dimeric model systems are particularly efficient since they provide double Lewis acid activation of the diester, thus enabling nucleophiles to attack the phosphorus center very readily.[4]

Since metal complexes have been found to be capable of catalyzing cleavage of model phosphodiesters, there has been considerable interest in making metal-containing artificial nucleases. Lanthanide(III), cerium(IV), zinc(II), or cobalt(III) complexes have been investigated for their ability to hydrolyze the phosphodiester bonds in DNA or RNA.[5] This work has been extended by workers attempting to make artificial restriction enzymes which will hydrolyze the DNA or RNA at specific sequences. With this approach, a metal center, often a lanthanide ion, that can hydrolyze the phosphodiester is attached to protein that can recognize a specific base sequence in the DNA or RNA. There has been some progress in making such artificial nucleases, with either Ln(III), Ce(IV), Cu(II), or Eu(III) complexes.[6]

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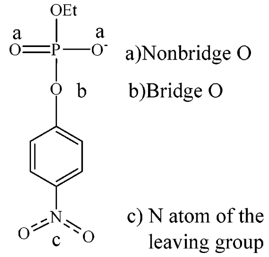

Figure 1. Positions of isotope effect measurements in EtpNPP.

Since metal ion catalysis of phosphodiester reactions is so common, a complete description of the transition state of phosphodiester hydrolysis catalyzed by a variety of metal ions should be obtained. One way of investigating the transition state of the reaction is through the study of the effects of isotopic substitution in the substrate on the reaction rate. Previous work[7] has looked at the hydrolysis of the model diester ethyl *p*-nitrophenyl phosphate (EtpNPP) catalyzed by complexes of Co(III), Zn(II), or Cu(II) or aqueous Eu(III). Although this diester has a good leaving group, which is not the case in DNA hydrolysis, extensive studies have been done using *p*-nitrophenyl esters,[8] and a complete study of all types of esters should be undertaken to show the range of mechanisms possible in phosphoester hydrolysis. Figure 1 shows the positions where isotopic substitution can be used in EtpNPP. The effects on the rate of substituting the ^{14}N in the nitrophenol group with ^{15}N were determined.[7] Among other results, it was found that Eu(III) showed an unusually large ^{15}N isotope effect with EtpNPP, which mirrored a large ^{15}N isotope effect observed with the Eu(III) or Er(III) catalyzed cyclization of uridine 3′-*p*-nitrophenyl phosphate.[9] Both of these measured isotope effects were larger than the equilibrium isotope effect for the dissociation of a proton from *p*-nitrophenyl phosphate (pNPP)[10] but were approximately of the same size as observed in a metaphosphate-like transition state for hydrolysis of pNPP.[11]

The effects of substituting ^{18}O for ^{16}O in the bridge position of the EtpNPP were obtained.[7] However, substantial additional information about bond changes in the transition state can be obtained by determining the effect on the rate of substitution of the nonbridge oxygens of the EtpNPP with ^{18}O. Thus, in this paper, the hydrolysis of isotopically substituted EtpNPP is investigated, using Co(III) or Zn(II) complexes and aqueous Eu(III) as model catalysts. To further investigate the unusual lanthanide effects, ^{15}N isotope effects were determined for the Ce(IV) catalyzed hydrolysis reaction of the diester EtpNPP and Ce(IV) and Eu(III) catalyzed reactions of the monoester pNPP. Figure 2 shows the ligands that were used in this investigation.

Experimental Section

Materials. 1,4,7,10-Tetraazocyclododecane (cyclen) sulfate, 1,5,9-triazacyclononane (tacn), disodium *p*-nitrophenyl phosphate, bis-Tris propane (BTP), $(NH_4)_2Ce(NO_2)_6$, and $Eu(NO_3)_3$ were obtained from Aldrich. Cyclen was purified by precipitation of the sulfate as barium sulfate and then recrystallization from hydrochloric acid. [Co(III) cyclenCl_2]Cl,[12] [Zn(II) cyclen]Cl_2[13] and [Co(III)$_2$ (tacn)$_2$ $(OH)_2$(EtpNPP)]-$(ClO_4)_3$[14] using the ethyl diester in place of the methyl diester, and nonlabeled EtpNPP[15] were synthesized by literature procedures. [^{15}N, nonbridge $^{18}O_2$]EtpNPP and ^{14}N-EtpNPP were synthesized[16] and then mixed together to reconstitute the natural abundance of 0.37% of ^{15}N.

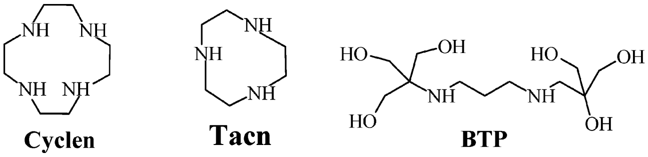

Figure 2. Ligands used for metal ion complexation.

Kinetics. Kinetic data were obtained on a Cary 300 spectrophotometer that was equipped with a Peletier temperature controller. Initial rates of the reaction were determined by following the increase in absorbance at 400 nm. Reaction conditions of 60 mM BTP, pH 8.5, and 1.67 mM phosphoester concentration duplicated the conditions of the isotope effect reactions.

Isotope Effect Sample Preparation. Fifty μmole samples of EtpNPP were used to determine R_o, the ^{15}N/^{14}N ratio in unreacted starting material. One hundred μmole samples of EtpNPP were used to begin IE experiments, which were stopped after partial conversion to determine R_p, the ratio of ^{15}N/^{14}N in the product, and R_s, the ratio of ^{15}N/^{14}N in the residual substrate. Enough [Co(III) cyclenCl_2]Cl was added to a 2.5 mM solution of EtpNPP equilibrated at 50 °C in 100 mM Hepes buffer, pH 7, to give a 10 mM final concentration of Co(III). [Zn(II) cyclen]Cl_2 was added to a 20 mM solution of EtpNPP equilibrated at 80 °C in 0.1 M EPPS, pH 8.5 so that the final Zn(II) concentration was 40 mM. $Eu(NO_3)_3$ was added to a 2 mM solution of EtpNPP in 50 mM MES, pH 6.3, 50 °C so that the final Eu(III) concentration was 10 mM. [Co(III)$_2$ (tacn)$_2$ $(OH)_2$(EtpNPP)]$(ClO_4)_3$, at 80 mM concentration, was dissolved in 0.1M Hepes, pH 7, equilibrated at 50 °C. $(NH_4)_2Ce(NO_2)_6$ was dissolved in a solution that was 60 mM in BTP, pH 8.5, to give a final Ce(IV) concentration of 3.33 mM. The phosphate compound was then added so the final phosphate concentration was 1.67 mM. Reactions were carried out at 25 °C for pNPP and 50 °C for EtpNPP. $Eu(NO_3)_3$ was dissolved in 36 mM BTP solutions, pH 8.5, to give a solution 10 mM in Eu(III), after which pNPP was added to a final concentration of 2 mM. The reaction was followed at 25 °C.

Isotope Effect Reactions. Reaction progress was monitored on a Cary 300 spectrophotometer at 400 nm. Reactions were quenched at approximately 50% conversion by adjusting the pH to 4. The product *p*-nitrophenol was then isolated by extracting the solution three times with an equal volume of distilled ether. To obtain the residual substrate, the reaction mixture was adjusted back to the initial pH and allowed to go to completion. This solution was once again extracted with ether after the pH was adjusted back to 4.

The ether solutions were dried with $MgSO_4$ and evaporated to dryness. The *p*-nitrophenol was then vacuum sublimed at 90 °C after which it was put in quartz tubes with copper and copper(II) oxide. The tubes were evacuated, sealed, and heated at 850 °C for 2 h. The nitrogen gas was separated from carbon dioxide and water vapor on a high vacuum line and collected on molecular sieves chilled with liquid nitrogen. The sieves were then heated to 200 °C to release the N_2 gas which was then analyzed on a Finnegan MAT Delta isotope ratio mass spectrometer.

Isotope Effect Measurements. The ^{15}N isotope effects were determined using the natural abundance of ^{15}N found in nitrophenol.

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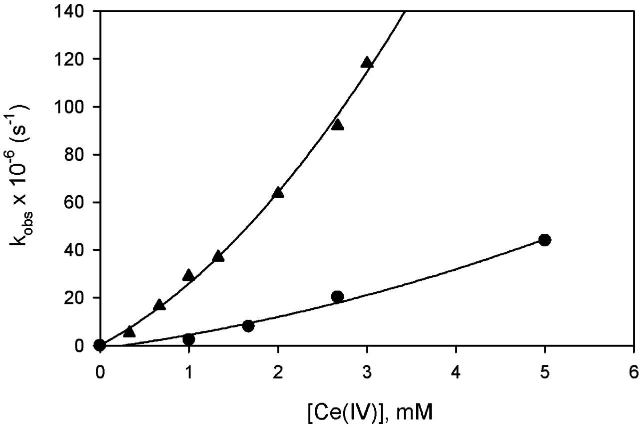

Figure 3. Dependence of k_{obs} on [Ce(IV)], 60 mM BTP, pH 8.5, circles; EtpNPP reaction, 50 °C; triangles, pNPP reaction, 25 °C.

The ^{18}O isotope effects were measured with the remote label method, using the nitrogen in the nitrophenol group as the reporter molecule. The ratios of $^{15}N/^{14}N$ in the product nitrophenol formed at ~50% reaction (R_p), in the residual substrate (R_s), and in the unreacted starting material (R_o) were determined by oxidation to N_2 and measuring the $^{15}N/^{14}N$ ration with an isotope ratio mass spectrometer. Isotope effects were calculated from the equations:

Isotope effect from residual substrate $= \log(1 - f)/\log[(1 - f)(R_s/R_o)]$ and

Isotope effect from product $= \log(1 - f)/\log[1 - f(R_p/R_o)]$,

where f is the fraction of reaction, and R_p, R_s, and R_o are defined above.

When used with a substrate that only contains natural abundance ^{15}N, these equations give the ^{15}N isotope effect. To obtain the ^{18}O isotope effect, substrate substituted with ^{18}O and ^{15}N is mixed with ^{16}O, ^{14}N substituted substrate so that the mixture is close to natural abundance in ^{15}N. After R_p, R_s, and R_o are determined with this mixture, the isotope effect is calculated using the above equations and then is corrected for the ^{15}N effect and for incomplete isotope incorporation.[17]

The isotope effect for a reaction carried out at 50 °C would be ~10% higher at 25 °C, and a reaction at 80 °C would have ~20% higher isotope effect at 25 °C.[10] This correction does not substantially change the values.

Results

Since kinetic data for the reaction of Ce(IV) with EtpNPP or pNPP have not been previously reported, Figure 3 shows how k_{obs} varies with Ce(IV) concentration in the presence of the solubilizing species BTP. The lines represent quadratic fits to the experimental data points.

Table 1 shows the measured isotope effects for the hydrolysis of EtpNPP catalyzed by Co(III) cyclen, Zn(II) cyclen, and Eu(III), and the hydrolysis of $[Co(III)_2(tacn)_2(OH)_2(EtpNPP)](ClO_4)_3$. The ^{15}N results reflect the effect of natural abundance ^{15}N on the reaction rate, while the ^{18}O results reflect isotopically substituted ^{18}O in either the bridge (leaving group) or nonbridge position of the EtpNPP. Table 2 shows the ^{15}N isotope effect for Ce(IV) or Eu(III) catalyzed reactions with pNPP or EtpNPP.

Discussion

Isotope effects have been used to characterize the transition states of many phosphate ester reactions.[8] The observation of a primary isotope effect for the bridge ^{18}O position indicates P—O bond fission is rate limiting, and the size of the effect is a measure of the degree of fission of the P—O bond in the transition state. For extensive bond fission in the leaving state, an isotope effect of ~1.03 may be expected, with smaller values expected for less advanced bond fission.[18] Protonation of the leaving group may also reduce this effect. Monoesters exhibit the largest values for this isotope effect, while the observed values for alkaline hydrolysis for bridge ^{18}O isotope effects for diesters with *p*-nitrophenol as the leaving group are in the range 1.0042−1.0063.[8]

As the P—O bond breaks, charge delocalizes into the nitrophenol leaving group, changing the bonding of the nitro group. If this step is rate limiting, a ^{15}N secondary isotope effect will be observed which is expected to be normal and proportional to the extent of bond cleavage in the transition state.

Secondary ^{18}O effects in phosphoesters arise from alterations in hybridization of the oxygens or from changes in the bending or torsional modes in the transition states. From the values observed for phosphoesters, changes in bond order dominate. Typical values for the nonbridge oxygen in diesters range from 1.0028−1.0056.[8]

Since a diester coordinated to two metal ions might be considered to more closely resemble a triester than a diester, it is relevant to consider the isotope effects measured for the related compound O,O-diethyl *p*-nitrophenyl phosphate. The measured bridge ^{18}O effect for the compound was 1.006, which together with the ^{15}N effect of 1.0007 was taken to mean that the phenolic oxygen bond order was about 0.75 in the transition state.[19] The secondary ^{18}O effect of 1.0063 was interpreted as resulting from a phosphoryl oxygen that had a bond order of 1.85 in the transition state, indicating that there was not very much bond loosening in the triester reaction and that the reaction proceeds with a fairly tight transition state.

Thus, a general model for diester hydrolysis shows a concerted transition state, with the bond between the phosphate and the nitrophenol breaking as the bond between the nucleophile and phosphate is forming. The isotope effects indicate P—O bond cleavage and loss of *p*-nitrophenol occur in the rate-determining step. The transition states for phosphodiesters (or triesters) are tighter, more associative than the transition state that is found in the hydrolysis of the dianion of the monoester *p*-nitrophenyl phosphate, which is looser and more dissociative.

Adding metal ions as catalysts for the phosphoryl transfer reaction presents the opportunity for altered mechanism. Such a change was observed for $[Co(III)_2(tacn)_2(OH)_2(EtpNPP)]^{3+}$, which seemed to react through formation of a discrete phosphorane intermediate.[20] We chose to look at other metal ion systems, to see how the presence of a metal ion would influence the reaction.

$[Co(III)_2(tacn)_2(OH)_2(EtpNPP)](ClO_4)_3$. Co(III) complexed to tacn can form stable complexes with phosphodiesters such as methyl *p*-nitrophenyl phosphate (MepNPP), which crystallize from acidic solution and have been shown to be dimeric, with a bridging phosphodiester and two bridging hydroxides,[21] as shown in Figure 4. Recent work has indicated that the reaction

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Table 1. Isotope Effects for the Reaction of EtpNPP with Metal Complexes

compound	^{15}N, nitro	^{18}O, bridge O	^{18}O,nonbridge O[a]
Co(III) cyclen[b]	1.0012 ± 0.0003	1.022 ± 0.001	0.9948 ± 0.0005
[Co(III)$_2$ (tacn)$_2$(OH)$_2$(EtpNPP)](ClO$_4$)$_3$	1.0026[c] ± 0.002	1.029[d] ± 0.002	1.0006 ± 0.0004
Zn(II) cyclen[e]	1.0005 ± 0.0003	1.0095 ± 0.001	1.0108 ± 0.0005
Eu(III)[f]	1.0034 ± 0.0004	1.016 ± 0.001	1.0016 ± 0.0007

[a] Isotope effects are reported as the overall effect from both O atoms. [b] Co(III) cyclen, (10 mM), EtpNPP (2.5 mM), pH 7 (0.1 M Hepes), 50 °C. [c] [Co(III)$_2$ (tacn)$_2$ (OH)$_2$(EtpNPP)](ClO$_4$)$_3$ (80 mM), pH 7 (0.1 M Hepes), 25 °C. [d] Reference 20. [e] Zn(II) cyclen (40 mM), Et pNPP (20 mM), pH 8.5 (0.1 M EPPS), 80 °C. [f] Eu(III) (10 mM), EtpNPP (2 mM), pH 6.3 (0.05 M MES), 50 °C.

Table 2. ^{15}N Isotope Effects of Ce(IV)- or Eu(III)-Catalyzed Reactions

metal catalyst	phosphate	^{15}N, nitro
Ce(IV) BTP[a]	EtpNPP	1.0017 ± 0.0004
Ce(IV) BTP[b]	pNPP	1.0014 ± 0.0004
Eu(III) BTP[c]	pNPP	1.0012 ± .0004

[a] Ce(IV), (3.33 mM), EtpNPP (1.67 mM), pH 8.5, (0.06 M BTP), 50 °C. [b] Ce(IV), (3.33 mM), pNPP (1.67 mM), pH 8.5, (0.06 M BTP), 25 °C. [c] Eu(III), (10 mM), pNPP, (2 mM, pH 8.6, (36 mM BTP), 25 °C.

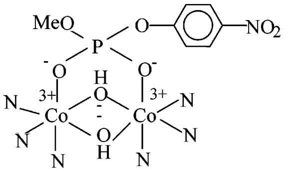

Figure 4. Structure of [Co(III)$_2$ (tacn)$_2$ (OH)$_2$(MepNPP)]$^{3+}$.

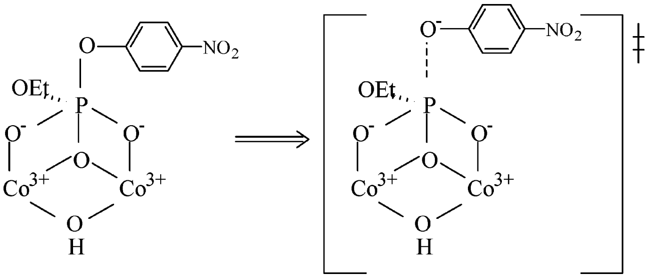

Figure 5. Phosphorane intermediate proceeding to the transition state.

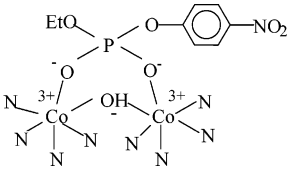

Figure 6. Structure of Co(III) cyclen + EtpNPP.

involves an oxide nucleophile bonding to the phosphorus resulting in a phosphorane intermediate.[20] Because there are ^{15}N and ^{18}O leaving group effects observed, the rate-limiting step is breakdown of the phosphorane intermediate, with release of *p*-nitrophenol, as shown in Figure 5. A concerted mechanism with an extremely productlike transition state is also a possibility. A negligible nonbridge isotope effect (1.0006 ± 0.0004) is observed for this reaction. The absence of a significant effect may be explained by changes in the bonding to the phosphoryl oxygens, which are compensated for by changes in Co—O bonding. As P—O bond order changes, Co—O interactions strengthen, and the bending modes are very restricted in this phosphorane-like transition state. Thus, an associative transition state might well not show the usual normal nonbridge isotope effect that an uncomplexed diester does.

Co(III) Cyclen + EtpNPP. The coordination sphere of [Co(III) cyclenCl$_2$]Cl consists of four nitrogen donors and two cis chlorides. Previous kinetic data showed the active species was dimeric, and the ^{31}P NMR indicated the diester was predominately dicoordinated to cobalt, as shown in Figure 6,[7] with some free diester in solution. The observed pH dependence of this reaction cannot distinguish between an oxide or a hydroxide nucleophile. The rate of the Co(III) cyclen reaction increases as pH increases to ~pH 7, reflecting the requirement for deprotonation, which would be observed with either nucleophile. With a metal-coordinated hydroxide nucleophile with pKa ~7.5, the rate would be expected to become constant at approximately pH 9.5, while the rate of an oxide nucleophilic reaction would keep increasing. However, the observed behavior shows the rate of the Co(III) cyclen decreases at pH > 7, with the decrease in rate above pH 7 most likely occurring because of less favorable phosphate ligand binding to the cobalt. However, analogous to the Co(III) tacn reaction, the only obvious nucleophile in the cyclen reaction would be the oxide nucleophile. Unlike the Co(III) tacn reaction, when Co(III) cyclen is dissolved in solution, both ligand coordination and phosphate transfer must occur.

Since coordination and phosphate transfer both occur, the observed nonbridge ^{18}O inverse isotope effect of 0.9948 would result from a primary ^{18}O equilibrium isotope effect on the formation of the cobalt(III) phosphate bond combined with the kinetic isotope effect from the phosphoryl transfer reaction $IE_{obs} = (IE_{transfer})\ (IE_{formation})$. Isotope effects for Co—O bond formation have been observed with related cobalt(III) complexes. With the monoester pNPP, bonding of the phosphate ligand to Co(III) cyclen is strong enough that shifts in the ^{31}P NMR and an equilibrium isotope effect of 0.9920 (the inverse of the observed isotope effect for dissociation) were found for complex formation.[22] An inverse equilibrium isotope effect indicates tighter binding for the nonbridge oxygens in products than reactants, which is reasonable, since in the complex, the nonbridge oxygens are bonded to some extent to Co(III). New bending modes are added and existing bends are compressed as a result of coordination to the metal center. With EtpNPP, a diester, weaker bonding might be expected to occur than with the more negatively charged monoester, and hence a smaller equilibrium isotope effect for the diester than the monoester would be expected when stretching modes predominate. Thus, the expected value for $IE_{formation}$ would be somewhat closer to 1 than the value of 0.9920 found for the pNPP reaction, and so the observed IE of 0.9948 probably can be assigned as the value of $IE_{formation}$. $IE_{transfer}$ would then be approximately 1, as found with the tacn complex.

The previously observed ^{15}N effect of 1.0012 for the Co(III) cyclen reaction with EtpNPP[7] reflects a significant negative charge that develops in the leaving group, and the bridge effect

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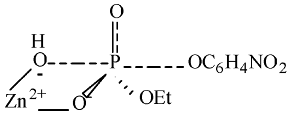

Figure 7. Transition state for Zn(II) cyclen reaction with EtpNPP.

of 1.022 indicates significant bond cleavage to the leaving group. The observed isotope effect confirms that the nitrophenol is lost in the rate-determining step with a large amount of bond cleavage at the transition state.

There are some differences in the measured isotope effects for the tacn vs cyclen complexes, and there are also differences between the four coordinate cyclen ligand and the three coordinate tacn ligand. With three available coordination positions, the tacn complex has two bridging hydroxides, which creates a relatively rigid core, with the phosphorus containing ligand positioned near the two bridging hydroxides, as shown in Figure 4. In the cyclen complex, however, with only two open coordination positions, only one hydroxide bridge can form. The inner metal core will not be quite so constrained and there could be a somewhat more open conformation to prevent nitrogen atoms of the two cyclen ligands from steric interaction. This “looser” structure then may explain the different oxygen isotope effects that are found with the cyclen compound.

Differences are also seen between the cyclen and tacn reactions with the monoester pNPP. With cyclen, the predominant species in a mildly acidic solution with Co(III) cyclen is the mono-coordinated pNPP, not a bridged monoester.[22] As reaction progresses, ^{31}P NMR signals characteristic of phosphate tricoordinated or tetracoordinated to Co(III) are seen with cyclen but not reported with tacn.[22,23] With the extra bridging hydroxide in the tacn complex, tri or tetracoordinated cobalt phosphate species may be too strained to persist in solution, while with the more flexible cyclen, such species can easily be identified with NMR.

Zinc(II) Cyclen + EtpNPP. Zn(II) cyclen acts as a monomer when added to a solution of EtpNPP.[7] Zinc(II) is generally considered to have more electrostatic bonding than cobalt (III). For instance, the equilibrium isotope effect for complexation of zinc(II) with *p*-nitrophenyl phosphorothioate was ∼1, indicating that the zinc(II) ligand interactions were weak and did not affect the *p*-nitrophenyl phosphorothioate bonding.[24] Other metals like magnesium(II) have also been reported to show insignificant isotope effects for metal complexation to phosphate.[25] Thus, the observed nonbridge bonding effect of 1.0108 likely results from the phosphoryl transfer reaction. The combination of a small ^{15}N effect, a primary bridge ^{18}O effect of ∼1.01, and the nonbridge ^{18}O effect indicates a concerted reaction with a later transition state and a larger amount of bond cleavage to the leaving group than found for the reaction of the uncomplexed diester with hydroxide,[24] as shown in Figure 7. The reaction would be more associative and would resemble the reactions of triesters.[20] The zinc(II) ion may be pulling electron density away from the nonbridge oxygen, resulting in changes in the transition state bonding. The zinc also generates an intramolecular nucleophile with a lower pKa than found for free hydroxide. Thus, the zinc(II)-catalyzed reaction occurs at much lower pH, with an appreciable rate at pH 8.5 for the Zn(II) reaction vs 1 N NaOH required for the uncomplexed reaction, but the weaker nucleophile in the metal-catalyzed reaction apparently causes significant differences in the transition state.

Europium(III) + EtpNPP, pH 6.3. The Eu(III) reaction shows a sigmoidal dependence of rate on Eu(III) concentration which likely indicates a dimeric complex is the active species.[7] Reactions must be done at relatively acidic pH (pH 6.3) because of limited solubility at higher pH. Work characterizing the reactions of Eu(III) and other lanthanides with bis(*p*-nitrophenyl) phosphate or diphenyl phosphate has found that the nitro group has little effect on reactivity,[26,27] suggesting the Eu(III) is coordinating the nonbridge oxygens. This possibility is supported by work with Ce(IV) which has shown significant interaction of the cerium with the phosphorus orbitals of model phosphodiesters.[28] However, such kinetic comparisons are based on a calculated rate constant for the uncatalyzed reaction of the extremely stable diphenyl phosphate, which does thus have some uncertainty. It would be difficult to explain the large ^{15}N effect, unless there was also some weak interaction of the europium ion with the nitro group, increasing electron flow into these oxygens, although the weak electron donating ability of the nitro group makes it an unlikely donor to the metal ion.[7]

From the very small nonbridge isotope effect of 1.0016 observed with Eu(III), one might conclude that the reaction was still concerted but with very little associative character and little change in the bonding of the nonbridge oxygen. The nonbridge IE of this reaction differs significantly from the nonbridge IE of the zinc reaction, suggesting a substantially different interaction of metal with ligand with Eu(III).

Europium(III) or Cerium(IV) Reactions, pH 8.5. In order to investigate the unusual ^{15}N isotope effects observed with Eu(III) catalyzed reactions of EtpNPP or uridine 3′-*p*-nitrophenyl phosphate,[9] reactions were investigated at a higher pH. In order to solubilize the metals, BTP was added. Previous work[29] has shown that Eu(III) forms dimeric systems with BTP and hydroxide ions, with a species formulated as $Eu_2(BTP)_2(OH)_2{}^{4+}$ predominating in solution around pH 8 before the phosphate ester is added. Thus, it is likely the hydrolysis reaction with Eu(III) involves a dimeric metal system with a hydroxide nucleophile. Reactions with Ce(IV) in BTP at pH 8.5 show a quadratic dependence on [Ce(IV)] for both the reaction with the monoester pNPP or the diester ethyl pNPP. A quadratic dependence on metal ion concentration was also found[29] in La(III) and Nd(III) reactions with bis(4-nitrophenyl) phosphate. In addition, dimeric or tetrameric species were found when Ce(IV) was reacted with BTP in acetonitrile before dilution in buffer.[30] These compounds were very effective catalysts for bis-(4-nitrophenyl) phosphate hydrolysis. A monomeric Ce(IV) is thus not the active species for the EtpNPP hydrolysis, and dimeric species are thus likely responsible for the rapid rates observed with Ce(IV) compared to metals such as Zn(II).

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The measured ^{15}N isotope effects with the Eu(III) or Ce(IV) at the higher pH are much more in line with other measured ^{15}N isotope effects, indicating that the unusual degree of charge delocalization into the nitrophenol group is not occurring under these conditions.

Conclusion

This work has shown that the reaction of the diester ethyl *p*-nitrophenyl phosphate proceeds very differently when complexed to a strongly coordinating metal such as Co(III) than when noncomplexed. Thus, instead of the usual isotope effects values of 1.0042−1.0063 found for the bridging oxygen, much larger isotope effect values of 1.029 and 1.022 are found with Co(III) tacn and Co(III) cyclen, indicative of far greater bond cleavage in the transition state, with bond fission in the Co(III) cyclen reaction somewhat less than in the Co(III) tacn reaction. The small nonbridge ^{18}O effect (1.0006) found in the Co(III) tacn reaction indicates little change in bonding to the nonbridge oxygen. The observed inverse isotope effect with the Co(III) cyclen reaction shows the covalent nature of the Co(III) complexes with phosphodiesters. Reactions with Zn(II) and Eu(III) differ from the reactions with Co(III), presumably reflecting the lack of covalent bonding between these metals and the diester as well as the monodentate nature of the complexation vs the bridging, bidentate binding of the phosphate ester in the binuclear cobalt complexes. The Zn(II) reaction resembles a triester reaction, with a relatively large perturbation of the nonbridge oxygen. Reaction with Eu(III) show little perturbation to the nonbridge O, perhaps suggesting the Eu(III) are binding to the nitro groups of the ligand. The Eu(III), at low pH, shows a large ^{15}N isotope effect because of delocalization of negative charge into the nitrophenol ring. This delocalization is reduced at more basic pH.

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