Characterization of Transition States in Dichloro(1,4,7-triazacyclononane)copper(II)-Catalyzed Activated Phosphate Diester Hydrolysis

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Abstract: The reaction mechanism for Cu[9]aneN_3Cl_2-catalyzed hydrolysis of ethyl 4-nitrophenyl phosphate was probed using kinetic isotope effects and isotope exchange experiments. The solvent deuterium isotope effect (^{D}k = 1.14), combined with the absence of ^{18}O incorporation into 4-nitrophenol, suggests that hydrolysis proceeds through intramolecular attack of the metal-coordinated hydroxide at the phosphorus center. The secondary ^{15}N isotope effect ($^{15}k = 1.0013 \pm 0.0002$) implies that loss of the leaving group occurs at the rate-limiting step with approximately 50% bond cleavage in the transition state. This study is one of the first applications of the secondary ^{15}N isotope effect to simple metal-promoted hydrolysis reactions, and the result is consistent with concerted bond formation and cleavage. A mechanism consistent with the isotope studies is presented.

Metal ions are essential cofactors in the reactions of many nucleases, and yet the precise role of the metal ion in the hydrolytic mechanism is unclear.[1] Similarly, many other biochemically important phosphoryl transfer reactions involve the action of metalloenzymes, frequently containing labile metal ions such as zinc(II) or magnesium(II).[2] The mechanism of metal complex-promoted phosphate diester hydrolysis has been explored in detail for substitutionally inert metal complexes of Ir(III),[3] but similarly detailed work has not been reported for labile metal systems involving Cu(II). There are several features of the reaction mechanism for labile metal-promoted phosphate diester hydrolysis which remain unclear, including the source of the nucleophile and the type of intermediate or transition state structure.

The nucleophile has been clearly identified for Ir(III)-promoted phosphate diester hydrolysis.[3] Through labeling studies, it was shown that phosphate diester hydrolysis occurred via an intramolecular attack of a metal-coordinated hydroxide at the phosphorus center. A similar mechanism has been proposed for labile metal complex-promoted phosphate diester hydrolysis.[4,5] Although intramolecular attack is the most commonly hypothesized mechanism, it is also possible that the metal-coordinated hydroxide could act as a general base to activate a solvent water molecule for attack at the phosphorus center.[6]

At present, there are few experimental data available to distinguish between metal-promoted phosphodiester hydrolysis via a pentacovalent phosphorane intermediate or via a concerted reaction with a phosphorane-like transition state structure. Metal complex-promoted hydrolysis of phosphate monoester monoanions and all phosphate diesters has been proposed to occur through the same S_N2-type mechanism as the alkaline hydrolysis of phosphate diesters.[7] A metal-bound hydroxide nucleophile has been proposed to attack the phosphorus center to form a pentacovalent phosphorane intermediate followed by rate-limiting loss of the leaving group,[8] but evidence for a phosphorane intermediate has been limited. Indirect evidence for such an intermediate has been obtained from labeling studies involving cobalt(III)-promoted hydrolysis of a bound phosphate monoester monoanion,[9] and a phosphorane intermediate has been invoked in the proposed mechanism for most inert[3,9] and labile[4,5] metal complexes. The generally proposed mechanism for phosphate diester hydrolysis has been challenged recently by studies of alkaline hydrolysis of activated phosphate diesters. The method of heavy atom isotope effects suggests that a concerted mechanism is operative.[10] Hydrolysis of an activated phosphodiester by the zinc(II)-dependent enzyme phosphodiesterase I proceeded with approximately 60% bond cleavage in the transition state, clearly implicating a concerted reaction path.[11] Although the authors attribute the efficacy of this enzyme to protonation of the phosphoryl group in the transition state, the role of the zinc ion in the catalysis could scarcely be assessed because of the paucity of relevant mechanistic data for metal-promoted hydrolyses.

Heavy atom isotope effects have been used for many years to study enzymatic reaction mechanisms.[12] Recently, the secondary ^{15}N isotope effect has been shown to provide a measure of the amount of bond cleavage in the transition state

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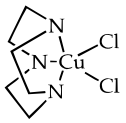
for phosphoryl transfer reactions.[10,13] Measurement of the secondary ^{15}N isotope effect on the alkaline hydrolysis of 3,3-dimethylbutyl 4-nitrophenyl phosphate revealed about 50% bond cleavage to the 4-nitrophenol leaving group in the transition state, corresponding to a concerted mechanism.[10] Application of the secondary ^{15}N isotope effect to metal complex-promoted hydrolysis of activated phosphate diesters will allow for the determination of whether the loss of the leaving group is rate limiting and, if so, will provide an estimate of the percentage of bond cleavage in the transition state. This information will improve our understanding of metal-mediated phosphodiester hydrolysis.

We have conducted detailed mechanistic studies of labile metal complex-promoted activated phosphate diester hydrolysis. Previously,[14] we described the hydrolytic activity of Cu[9]aneN_3-Cl_2 (**1**), an effective catalyst for phosphate diester hydrolysis.[15] In order to probe the mechanism in greater detail, a series of isotope effect studies have been performed. To obtain information on the source of the nucleophile, the solvent deuterium isotope effect on the rate of **1**-catalyzed phosphate diester hydrolysis has been determined. To assist in the determination of the rate-limiting step as well as to distinguish between a pentacovalent phosphorane intermediate and a concerted mechanism, the secondary ^{15}N isotope effect for the hydrolysis of ethyl 4-nitrophenyl phosphate (**2**) by **1** has been measured. These studies have enabled us to develop a more complete understanding of the mechanism of metal-promoted hydrolysis.



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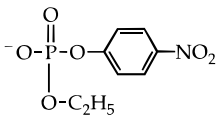

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Experimental Methods

Materials. The biological buffers HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid) and CHES (2-(*N*-cyclohexylamino)-ethanesulfonic acid) were purchased from Sigma Chemical. Chelex resin (sodium form) was also purchased from Sigma Chemical. The isotopically labeled compounds D_2O (99.99+%) and $^{18}OH_2$ (60−65%) were purchased from Cambridge Isotope Laboratories. All chemicals were used without further purification. Anhydrous analytical grade diethyl ether (Mallinckrodt) was distilled at 34−35 °C to remove higher boiling impurities shortly before use. Sodium ethyl 4-nitrophenyl phosphate and dichloro(1,4,7-triazacyclononane)copper(II) were prepared as described.[14] All aqueous solutions were prepared with water purified by passage through a Millipore purification system. The instrumentation was as described.[14]

Kinetic Procedures. The initial rate of production of 4-nitrophenolate was monitored spectrophotometrically at 400 nm as described.[14] All experiments were run in triplicate, and the data reported represent the average of these experiments with less than 5% deviation among the measurements.

The effect of temperature on the hydrolysis of **2** by the catalyst derived from **1** was determined over the temperature range 30−90 °C. Reactions were performed in 50 mM CHES buffer, the pH of the buffer was adjusted to 9.1 at each reaction temperature, and the reaction mixture contained 1.00 mM **1** and 10.0 mM **2**. The ionic strength was 0.1 M and was controlled with 5 M $NaClO_4$. Reactions were prepared in parallel to insure identical conditions of pH, concentration, and ionic strength and were pre-equilibrated at the desired temperatures. Four different temperatures were used: in one experiment, the actual temperatures used were 32.0, 50.2, 65.3, and 84.0 °C. The measured temperatures are accurate to ±0.5 °C. To maintain a constant temperature at 32 °C, an external ice bath was used to cool the water circulator.

The solvent deuterium isotope effect for the hydrolysis of **2** by **1** was determined. Reactions were performed in H_2O or 99.99% D_2O at pH > 9.0, corresponding to the pH-independent region of the pH vs rate profile,[14] in order to eliminate any rate effect due to differences between pH and pD.[16] As a control, the reaction was run in CHES buffer at pH ± 0.5 from the calculated pD, and the ^{D}k was determined to be constant. In a typical experiment, freshly prepared **1** stock (11 mM) in either H_2O or D_2O was added to a reaction mixture containing 50 mM CHES buffer (pH or pD > 9.1) in the same solvent and heated to 50 °C in the spectrophotometer. A reference cuvette, identical in all respects except lacking **1**, was similarly prepared. Freshly prepared **2** stock (51 mM) in the appropriate solvent was added to the reaction mixtures, and the mixtures were allowed to equilibrate for 5 min. The reactions in D_2O and H_2O were carried out in parallel under identical conditions. The total reaction volume was 3.00 mL, the final concentration of **1** was 1.17 mM, the final concentration of **2** was 2.57 mM, and the ionic strength was adjusted to 0.1 M with 5 M $NaClO_4$.

Isotope Incorporation Determination. The ^{18}O isotope incorporation in the hydrolysis of **2** by the catalyst derived from **1** was measured. The reaction mixture consisted of 50 mM **2**, 10 mM **1**, and 30% $^{18}OH_2$, and the pH was held constant at 7.2 with 0.5 M HEPES. The solution was heated at 70 °C in a heat block for 3 weeks, at which time the reaction had reached about 40% completion, as determined by the amount of 4-nitrophenolate formed. The 4-nitrophenol was isolated by acidification followed by ether extraction. The ether layer was dried over magnesium sulfate, and the ether was removed by rotary evaporation. The resulting yellow residue of 4-nitrophenol was dissolved in 400 μL of D_2O, and its identity was confirmed by ^1H NMR. The D_2O was removed by rotary evaporation, and the sample of 4-nitrophenol was submitted for high-resolution mass spectrometry to measure ^{18}O incorporation.

Secondary ^{15}N Isotope Effect Determination. The secondary ^{15}N isotope effect for the hydrolysis of **2** by **1** was determined with the natural abundance of nitrogen in the substrate by the method of Hengge and Cleland.[10] For these experiments, reactions involved isolation and measurement of the $^{15}N/^{14}N$ ratio for 4-nitrophenol as described.[17] Specifically, the reaction mixture contained 5 mM **2**, 1.25 mM **1**, and 20 mL of 0.1 M HEPES buffer, pH adjusted to 7.2. The reaction mixtures were heated and stirred at 70 °C in an oil bath. After the reaction reached 40−60% completion, the reaction was stopped and the amount of 4-nitrophenol produced was determined. The reaction mixture was acidified with 1 M HCl and the 4-nitrophenol extracted three times with distilled diethyl ether. The ether extracts were dried over magnesium sulfate and concentrated to dryness. The aqueous layer, containing the unreacted substrate as well as **1**, was subjected to rotary evaporation to remove dissolved ether. To prevent interference with the ^{15}N measurement, **1** was removed by passage through a 5 mL Chelex column, and the column was washed with 20−25 mL of H_2O to ensure recovery of all the unreacted substrate. The eluent was hydrolyzed completely by the addition of enough NaOH to make a 1 M solution, followed by heating at 95 °C. Complete hydrolysis was ensured by heating the basic solution for at least 30 h, which is 10 times the half-life for base hydrolysis at this pH and temperature. The 4-nitrophenol produced was isolated as before.

The purification and preparation of the 4-nitrophenol samples for combustion was performed by the method of Cleland.[17] Briefly, the 4-nitrophenol residues were purified by sublimation under vacuum at 95 °C, and the sublimed product was transferred to a quartz tube by rinsing with distilled diethyl ether. The ether was removed from the tubes under vacuum, and the tubes were prepared for combustion by the addition of a layer of CuO, a layer of copper metal, and a thin piece of silver foil. The samples were combusted at 850 °C for 2 h, then at 550 °C for 8 h before cooling to room temperature.

After combustion, the nitrogen gas was separated from the water vapor and carbon dioxide on a high-vacuum line by selective trapping of the latter two gases with dry ice/alcohol and liquid nitrogen traps, respectively. The nitrogen gas was deposited onto molecular sieves cooled with liquid nitrogen and then released from the molecular sieves by heating to 200 °C for 15 min before transfer to the isotope ratio mass spectrometer where the isotopic composition of the N_2 gas was

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Table 1. Activation Parameters for Phosphate Ester Hydrolysis[a]

nucleophile	substrate	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ [b] (kJ mol^{-1})	k_{calcd} [c] $\times 10^6$ s^{-1}	ref
[9]Cu-OH$^+$	**2**	85	−79	108	0.58	*d*
(en)$_2$Ir-OH^{2+}	**2**	69	−93	97	70	3
(en)$_2$Co-OH^{2+}	NPP	71	−65	90	900	9
OH$^-$	BDNPP	80	−107	111	0.15	24

[a] Abbreviations: [9],1,4,7-triazacyclononane; en, ethylenediamine; NPP, 4-nitrophenyl phosphate; BDNPP, bis(2,4-dinitrophenyl) phosphate. [b] $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, calculated at 25 °C. [c] Rate constants calculated at 25 °C for given activation parameters. [d] Activation parameters calculated from $k_{obsd} = \nu$/[LCu], where [LCu] is defined as previously.[14]

determined. The hydrolysis of **2** by **1** was run in two triplicate sets, and the resulting values were averaged.

To determine the isotopic composition of the starting material, 50 μmol of **2** was placed in 1 M NaOH and heated at 95 °C for 30 h to ensure complete hydrolysis. The 4-nitrophenol was extracted and converted to nitrogen gas and the isotopic composition determined as described above (*vide supra*). The isotopic composition of the starting material was also determined by direct combustion of 5 mg of solid **2**. The isotopic ratios were identical within experimental error, and the average was used.

The isotope effects were calculated using the isotopic ratio obtained from the isotope ratio mass spectrometer. The three sets of isotope ratios obtained were the product at partial reaction (R_p), the residual substrate (R_s), and the starting material (R_o). Measuring the isotopic ratio of both the product at partial completion and the residual substrate allowed two independent calculations of the isotope effect for each experiment, one involving R_p and R_o and one using R_s and R_o.[18]

Results

Computation of Activation Parameters. To determine the effect of **1** on the magnitude of the activation barrier for the hydrolysis of **2**, the activation parameters were measured in the pH-independent region of the previously determined pH vs rate profile.[14] The initial rate data were analyzed by use of the modified Arrhenius equation. The plot of ln initial rate versus $1/T$ was linear, yielding $\Delta H^{\ddagger} = 85 \pm 1.7$ kJ mol^{-1} and $\Delta S^{\ddagger} = -143 \pm 5$ J mol^{-1} K^{-1}. These values were determined over a limited temperature range; the error in the experimental determinations is small, but extrapolation beyond this temperature range may not be valid.

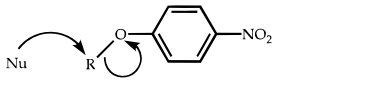
In order to compare the measured activation parameters to reported values, the parameters were calculated from the first- and second-order rate constants. The rate constants were calculated by applying the experimental reaction orders previously found for **1** (half-order) and **2** (first order) to the experimentally measured initial rate.[14] The magnitude of $\Delta H^{\ddagger}$ is independent of reaction order, but $\Delta S^{\ddagger}$ is dependent on the reaction order.[19] If the initial rates of reaction are converted to k_{obsd} by removing the **1** dependence, then $\Delta H^{\ddagger} = 85 \pm 1$ kJ mol^{-1} and $\Delta S^{\ddagger} = -79 \pm 1$ J mol^{-1} K^{-1}. Since the reaction was catalytic in **1** and there was a 10-fold excess of **2** to **1**, the concentration of **1** is negligible; therefore, this calculation gives a reasonable value for $\Delta S^{\ddagger}$. If the initial rates of reaction are converted to second-order rate constants by removing both the **1** and **2** dependencies, then $\Delta H^{\ddagger} = 85$ kJ mol^{-1} and $\Delta S^{\ddagger} = -40$ J mol^{-1} K^{-1}.

Relatively few workers have reported activation parameters for metal complex-promoted phosphate diester hydrolysis. A compilation of the reported values for metal complex-promoted phosphate diester, metal complex-promoted phosphate monoester, and alkaline phosphate diester hydrolyses is given in Table 1.

Solvent Deuterium Isotope Effect Analysis. The solvent kinetic isotope effect $^{D}k^{20}$ was determined in order to distinguish between a nucleophilic reaction path and a general base reaction path. A general base mechanism is usually associated with a solvent deuterium isotope effect of >2, while a nucleophilic mechanism is usually associated with an isotope effect ranging from 0.8 to 1.5.[6] The solvent isotope effect observed for **1**-catalyzed hydrolysis of **2** is $^{D}k = 1.14$, for reactions carried out under otherwise identical conditions. This value is consistent with the intramolecular attack of a coordinated nucleophile where proton transfer does not occur in the rate-determining step. Table 2 lists published deuterium solvent isotope effects for various phosphate ester hydrolyses for comparison with this measured value.

^{18}O Isotope Incorporation Determination of Bond Cleavage Site. Nucleophilic attack at the phosphorus center is the most common mode of phosphate diester cleavage, but nucleophilic attack at the aromatic carbon is also possible. For example, the alkaline hydrolysis of methyl 2,4-dinitrophenyl phosphate occurred by a mixed mechanism where both C−O and P−O bond cleavages were observed.[21] For the hydrolysis of **2** by **1**, mass spectral analysis of the 4-nitrophenol indicated no incorporation of ^{18}O. The upper limit of ^{18}O incorporated into the sample was 2%, well within experimental error.

Secondary ^{15}N Isotope Effect Analysis. The secondary ^{15}N isotope effect can provide a direct measure of the transition state bond cleavage of phosphate esters with 4-nitrophenol leaving groups.[10] The effect arises from the resonance stabilization of the partial negative charge resulting from partial cleavage of the bond to the leaving group, through contribution from the quinoid resonance structure, **4**. Nitrogen bonds to oxygen are



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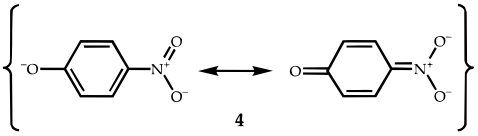

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more stiffening than nitrogen bonds to carbon in terms of vibrational frequencies, and the ^{15}N label will enrich in the more stiffly bonded neutral species. A normal isotope effect (value greater than 1.0000) is therefore expected for the formation of the 4-nitrophenolate anion.

The isotope effect was calculated from the ratio of masses obtained from the isotope ratio mass spectrometer by the literature method.[18] A measure of the precision of the experiment can be obtained by comparing the isotope effect calculated from R_p and R_o to the isotope effect calculated from R_s and R_o.

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Table 2. Solvent Deuterium Isotope Effect for Phosphate Ester Hydrolysis[a]

nucleophile	substrate	$^{D}k^{b}$	mechanism[c]	ref
[9]Cu-OH$^+$	**2**	1.14[d]	n	this work
OH$^-$	BDNPP	1.55	?	24
(en)$_2$Co-OH^{2+}	NPP	1.20	n	9
pyridine	MDNPP	1.0	n	21
acetate ion	MDNPP	1.1	n	21

[a] Abbreviations: [9],1,4,7-triazacyclononane; en, ethylenediamine; NPP, 4-nitrophenyl phosphate; BDNPP, bis(2,4-dinitrophenyl) phosphate; MDNPP, methyl 2,4-dinitrophenyl phosphate. [b] The isotope effect notation used is that of Northrop,[20] in which isotope effects are shown by a leading superscript. The kinetic isotope effect k^H/k^D is written as ^{D}k, where D corresponds to deuterium. [c] Mechanism assigned by criteria described in the text: n, nucleophilic; ?, not conclusive. [d] Ratio of ν_H/ν_D measured under identical conditions of metal complex concentration, substrate concentration, temperature, and pH.

Table 3. Comparison of the Secondary ^{15}N Isotope Effect for Hydrolysis of Alkyl 4-Nitrophenyl Phosphate by a Series of Nucleophiles[a]

hydrolytic system[b]	$^{15}k^{c}$	approx % bond cleavage[d]
[9]Cu-OH$^+$	1.0013 ± 0.0002	46
OH$^-$	1.0016 ± 0.0001	57
H$^+$	1.0009 ± 0.0002	32
β-cyclodextrin	1.0013 ± 0.0001	47
phosphodiesterase I	1.0017 ± 0.0002	61

[a] Reported errors are standard errors. [b] Data for comparison with [9]Cu-OH$^+$ are from Hengge and Cleland.[11] [c] The isotope effect notation used is that of Northrop,[20] in which isotope effects are shown by a leading superscript. The kinetic isotope effect k^{14}/k^{15} is written as ^{15}k, where 15 denotes ^{15}N. [d] The percentage of bond cleavage in the transition state is calculated relative to a standard reference according to the method of O'Leary.[18] The use of this standard and the calculation method assume a value of $^{15}k = 1.0028$ to represent complete bond cleavage in the transition state (see text) and assume a linear relation between isotope effect and the fraction of bond cleavage.

The two values are expected to be the same, and the values reported here are consistent with one another to within experimental error. The overall isotope effect of $^{15}k = 1.0013 \pm 0.0002$ was obtained by averaging the isotope effects calculated for both R_s, and R_p for a total of six experiments. A comparison with secondary ^{15}N isotope effects for phosphate diester hydrolyses in several other systems is given in Table 3.

Discussion

Effect of Metal Complexes on the Activation Parameters. There has been no attempt in the literature to interpret the activation parameters for metal complex-promoted phosphate diester hydrolysis, and the relatively few parameters that have been reported are compiled in Table 1. The complexity of hydrolysis reactions combined with the large number of species in solution prohibit extensive interpretation; however, several generalizations are noted. There is a considerable range of values observed for both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ but no trend is evident. In all cases, $\Delta S^{\ddagger}$ is less than zero as expected for bringing together multiple reactive species in solution. The free energy barriers, $\Delta G^{\ddagger}$ for various phosphate diester hydrolyses have been calculated from the reported $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values and are listed in Table 1. From the ratio of $\Delta G^{\ddagger}$s for the hydrolysis of **2** by **1** or (en)$_2$Ir-OH^{2+}, it is noted that an increase in $\Delta G^{\ddagger}$ of about 5 kJ mol^{-1} results in a 10-fold decrease in the rate constant. The presence of a metal complex lowered $\Delta G^{\ddagger}$ for phosphate diester hydrolysis, as would be predicted for any compound which promotes a reaction.

In the case of hydrolysis of **2** by **1**, it should be noted that the initial rates measured reflect the composite effect of temperature on four distinct processes: the monomer−dimer equilibrium, the protonation constant for the coordinated water, substrate exchange on the catalyst, and the hydrolysis reaction. The forward and backward rates in the monomer−dimer equilibrium, as well as the substrate exchange and the deprotonation steps, are expected to be fast, largely influenced by rapid proton transfer and ligand exchange rates. Ligand exchange on Cu(II) typically occurs with rate constants of 10^8 s^{-1}.[22] Exchange rates and activation parameters for solvent exchange on Cu(MeOH)$_6^{2+}$ have been reported; an exchange rate of $k_{exch} = 3 \times 10^7$ s^{-1} (at 25 °C) and a $\Delta H^{\ddagger} = 17$ kJ/mol were measured for this system.[23] For the purposes of the following qualitative comparisons, we have assumed that since loss of the leaving group is rate limiting in hydrolysis of **2** by **1**, the measured temperature dependence is largely due to this step in the reaction mechanism.

It is of interest to determine the extent to which the presence of **1** decreases the free energy barrier to hydrolysis of **2**. To directly compare the activation energy for **1** hydrolysis of **2** to that for alkaline hydrolysis of **2**, it is necessary to estimate the energy barrier for alkaline hydrolysis of **2**. The rate of this reaction is sufficiently slow that temperature dependence studies are not feasible; the data available in the literature is for the alkaline hydrolysis of bis(2,4-dinitrophenyl) phosphate.[24] In general, the rate constant for the hydrolysis of activated phosphate diesters increases with a more acidic pK_a of the leaving group;[21,24] therefore, the $\Delta G^{\ddagger}$ for alkaline hydrolysis of bis(2,4-dinitrophenyl) phosphate is expected to be smaller than the activation barrier for alkaline hydrolysis of **2**, since 2,4-dinitrophenol is more acidic than 4-nitrophenol. The rate constant for alkaline hydrolysis of **2** was estimated as 10^{-9} s^{-1} at 25 °C by Hendry and Sargeson,[3] and therefore, $\Delta G^{\ddagger}$ is expected to be 10 kJ mol^{-1} higher than that for bis(2,4-dinitrophenyl) phosphate. From this data we estimate $\Delta G^{\ddagger}$ to be 120 kJ mol^{-1} for the alkaline hydrolysis of **2**.

The $\Delta G^{\ddagger}$ estimated for the alkaline hydrolysis of **2** is compared to the hydrolysis of **2** by **1** or (en)$_2$Ir-OH^{2+}. The presence of the catalyst, **1**, causes an estimated decrease in $\Delta G^{\ddagger}$ of 12 kJ mol^{-1} relative to the estimated $\Delta G^{\ddagger}$ for alklaine hydrolysis of **2**. The corresponding increase in the rate constant is calculated to be 10^2, approximately the same magnitude as the rate increase that was observed previously[14] for the hydrolysis of bis(4-nitrophenyl) phosphate by **1** when compared to the alkaline hydrolysis of the same substrate at comparable concentrations of **1** and hydroxide. A decrease in $\Delta G^{\ddagger}$ of 22 kJ mol^{-1} is noted for (en)$_2$Ir-OH^{2+}-promoted hydrolysis of **2** compared to the alkaline hydrolysis of **2**. Although the decrease in $\Delta G^{\ddagger}$ is consistent with the preorganization of the metal-bound substrate, the entropic contribution is about the same as that found in **1** hydrolysis of **2**. Preorganization of the nucleophile and substrate in the metal complex is expected to result in a less negative $\Delta S^{\ddagger}$; however, no difference in the entropic effect is observed. Interestingly, a substantial $\Delta H^{\ddagger}$ decrease is observed for (en)$_2$Ir-OH^{2+}-promoted hydrolysis of **2** compared to **1** hydrolysis of **2**. The 3+ charge on the metal as well as the preorganization of the complex in an orientation favorable for nucleophilic attack presumably contributes to lower the overall activation enthalpy. The $\Delta G^{\ddagger}$ for (en)$_2$Ir-OH^{2+}-promoted hydrolysis of **2** is 10 kJ mol^{-1} less than the $\Delta G^{\ddagger}$ for **1**-catalyzed hydrolysis of the same substrate, suggesting that the overall thermodynamic contribution of the Ir(en)$_2^{3+}$ complex is substantial. The lower activation barrier corresponds to a 2 orders of magnitude increase in the rate constant of Ir(III)

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compared to the rate constant of **1**. Assuming that the lowering of the activation barrier can be assigned to the combination of preorganization of the metal−substrate complex and more effective charge neutralization, approximately 2 orders of magnitude of the 6×10^{-7} s^{-1} rate constant measured for **1**-catalyzed hydrolysis of **2** can be assigned to the equilibrium between the catalyst and the phosphate diester substrate. The remaining value of 6×10^{-5} s^{-1} can be attributed to the hydrolysis of the phosphate diester.

Intramolecular versus Intermolecular Nucleophilic Attack. In general, intramolecular nucleophilic attack has been associated with large rate accelerations due to the increased order of the system.[25] Intramolecular attack of a coordinated hydroxide at the phosphorus center has been conclusively demonstrated for phosphate diester hydrolysis by substitutionally inert metal complexes of Co(III) and Ir(III).[3,9] A similar mechanism has been proposed for Cu(2,2′-bipyridine)$^{2+}$-catalyzed hydrolysis of **2**, but no direct evidence was reported.[4]

A classic method for probing the difference between a nucleophilic mechanism and a general base mechanism is to measure the solvent deuterium isotope effect.[6] For the reaction of **1** with **2**, the two possible reaction pathways are either direct nucleophilic attack by the coordinated hydroxide at the phosphorus or nucleophilic attack at the phosphorus by an activated water molecule. Since no transferable proton will be involved in the rate-limiting step for the former mechanism, a normal solvent deuterium isotope effect will be observed only for the latter case. The measured ^{D}k of 1.14 for **1**-catalyzed hydrolysis of **2** (Table 2) implies that no proton transfer is involved in the rate-limiting step. This observation suggests that the mechanism involves intramolecular nucleophilic attack by a copper(II)-coordinated hydroxide. An alternative explanation, that an inverse isotope effect on a different step in the reaction mechanism is obscuring a large normal isotope effect, can be ruled out by the following considerations. As mentioned previously, exchange rates on Cu(II) are much more rapid than hydrolysis; therefore, kinetic isotope effects on processes dominated by ligand exchange, such as the monomer−dimer equilibrium, will not be measured in this experiment. The same argument holds true for proton transfers. Any equilibrium solvent isotope effect on the protonation constant for the metal complex is eliminated by carrying out the reactions well above the pK_a of the coordinated water. An equilibrium solvent isotope effect on the monomer−dimer equilibrium is possible, although the monomer−dimer interconversion most reasonably occurs via ligand dissociation or aquation and not proton transfer.

The solvent deuterium isotope effect strongly implicates an intramolecular reaction, with attack of the coordinated hydroxide on the phosphorus atom. Such isotope effects have not been measured previously for labile metal complex-promoted phosphate diester hydrolyses. Solvent deuterium isotope effects that have been measured for organic nucleophiles used to promote phosphate ester hydrolysis indicate an intramolecular nucleophilic mechanism.[21] The solvent isotope effect for hydrolysis of the coordinated 4-nitrophenyl phosphate in the metal complex Co(en)$_2$(4-nitrophenyl phosphate)(OH)$^+$ has been measured and was also assigned to an intramolecular nucleophilic mechanism.[9] Interestingly, the alkaline hydrolysis of bis(2,4-dinitrophenyl) phosphate gave a solvent deuterium isotope effect of 1.55, a value which the authors assigned to a general base mechanism,[24] although this value is lower than that usually found for general base reactions.[6] A solvent deuterium isotope effect in the range 1.5−2.0 is generally considered inconclusive for either type of mechanism.

Position of Bond Cleavage. While the solvent deuterium isotope effect indicates that the reaction occurs by nucleophilic attack of the coordinated hydroxide, the solvent isotope effect does not distinguish between attack at phosphorus and attack at the aromatic carbon. Although attack at the aromatic carbon appears unlikely, nucleophilic aromatic substitution at a ring carbon has been observed in the hydrolysis of other aryl phosphate esters.[21] To gain information on the site of bond cleavage, the hydrolysis of **2** by **1** was run in the presence of $^{18}OH_2$. If the coordinated hydroxide attacks at the phosphorus center, then the ^{18}O label will not be incorporated into the 4-nitrophenol product. On the other hand, coordinated hydroxide attack at the aromatic carbon will result in ^{18}O label incorporation into 4-nitrophenol. The insignificant quantity of ^{18}O detected by mass spectral analysis of the 4-nitrophenol produced by **1**-catalyzed hydrolysis of **2** indicates that the reaction proceeded essentially completely through attack at the phosphorus with the corresponding P−O bond cleavage, with negligible or no competing C−O bond cleavage.

Secondary ^{15}N Isotope Effect. The generally postulated mechanism for activated phosphate diester hydrolysis by a metal complex involves the formation of a pentacovalent phosphorane intermediate followed by the rate-limiting loss of the leaving group. Although attempts have been made to detect the phosphorane intermediate, all have been unsuccessful.[3,9] Instead of directly detecting the intermediate, the reaction mechanism can be probed at the rate-limiting step with the secondary ^{15}N isotope effect.[10,26]

Since an isotope effect will only be observable to the extent that the isotopically sensitive step is rate limiting, the presence or absence of an observed ^{15}N isotope effect will give mechanistic information. For metal complex-promoted phosphate diester hydrolysis with a good leaving group like 4-nitrophenol, the mechanism can be associative or concerted. In an associative mechanism, the formation of a pentacovalent phosphorane intermediate is rate limiting, followed by the rapid loss of the activated leaving group. Since the loss of 4-nitrophenol will not be involved in the rate-limiting step, no secondary ^{15}N isotope effect is expected. A concerted reaction mechanism, where the bond between the phosphate and the 4-nitrophenol is breaking at the same time as the bond between the nucleophile and the phosphate is forming, will yield a normal secondary ^{15}N isotope effect proportional to the extent of bond cleavage in the transition state. A dissociative mechanism is considered unlikely for phosphodiester hydrolysis, even in the presence of an activated leaving group.[11]

Previous work on the alkaline hydrolysis of 4-nitrophenyl phosphate suggests that the reaction is concerted with a dissociative transition state.[27,28] The secondary ^{15}N isotope effect for the alkaline hydrolysis of 4-nitrophenyl phosphate $^{15}k = (1.0028)$ is the largest observed, supporting the earlier conclusion that bond cleavage to the leaving group is far advanced in the transition state.[10] The maximum ^{15}k, representing complete bond cleavage in a dissociative mechanism, is unknown, but the large value observed for alkaline hydrolysis of 4-nitrophenyl phosphate has been used as a standard, representing virtual total bond cleavage.[10] This reference reaction is a phosphate monoester hydrolysis, and while it is not ideal for comparison with phosphate diester hydrolyses, it is the best that is available. Calculating the percentage of bond cleavage at the transition state relative to the alkaline hydrolysis of 4-nitrophenyl phosphate therefore provides a lower limit on

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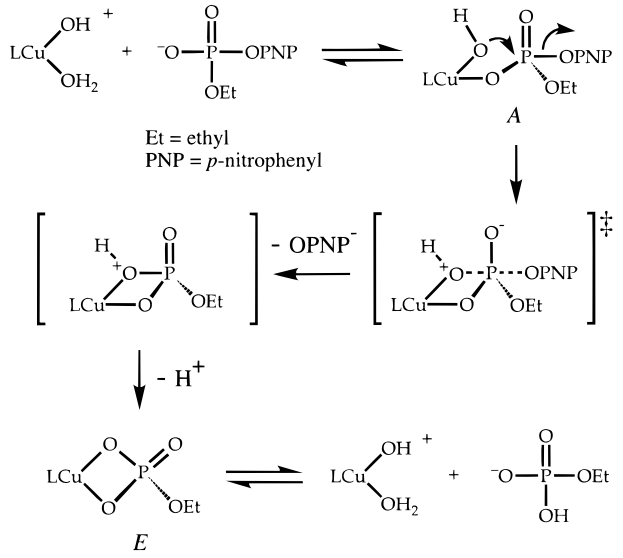
Scheme 1

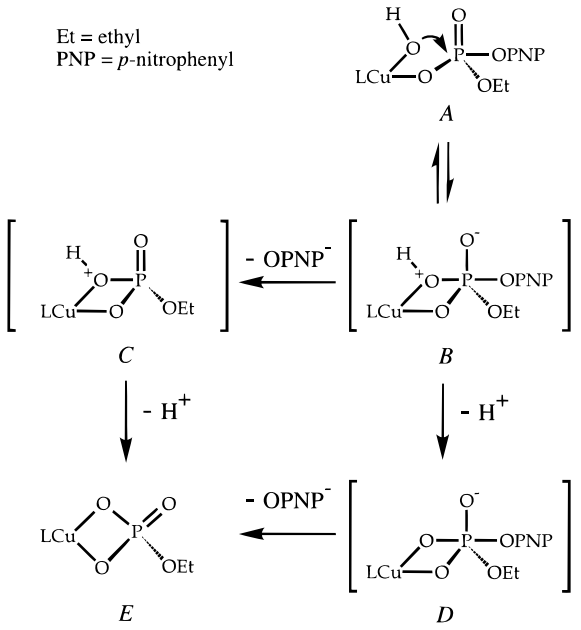


the extent of bond cleavage.[10,11] The calculation assumes a linear relationship between isotope effect and the fraction of bond cleavage; this model provides the best current method to evaluate the data, albeit in the absence of experimental validation. For **1**-catalyzed hydrolysis of **2**, the secondary ^{15}N isotope effect was measured as 1.0013 ± 0.0002. The presence of a normal isotope effect confirms that the loss of the leaving group occurs at the rate-limiting step. In the transition state for the hydrolysis of **2** by **1**, the leaving group bond cleavage is about 50%, and this value corresponds best to a concerted mechanism, as discussed in detail below.

Mechanistic Analysis. The isotope effect studies discussed above, combined with the kinetic data reported previously,[14] enable us to evaluate the detailed mechanism for **1**-catalyzed hydrolysis of activated phosphate diesters. The mechanistic possibilities, illustrated in Schemes 1 and 2, are now discussed in detail. The catalytically active copper(II) hydroxide complex binds directly to the phosphate diester to form *A*, positioning the coordinated hydroxide for intramolecular nucleophilic attack at the phosphorus center. One possibility is that species *A* may react via nucleophilic attack simultaneous with leaving group departure in a concerted mechanism, followed by rapid deprotonation to give *E*. This mechanism, which we believe to be operative, is shown in Scheme 1. There is no proton in flight in the rate-determining step, consistent with the near unity value of ^{D}k observed. Partial cleavage of the bond to the leaving group is in agreement with the observed moderate value for ^{15}k.

An alternative possibility is an associative mechanism via a phosphorane intermediate. This mechanism can be ruled out by careful consideration of the relative rates of competing processes and the observed kinetic isotope effects, ^{D}k and ^{15}k. The species initially formed upon intramolecular nucleophilic attack of the metal-bound hydroxide is shown as *B* in Scheme 2. The hypothetical phosphorane *B* could partition either of two ways depending on the relative rates of proton loss from the bridging oxygen atom and competing loss of *p*-nitrophenolate. Given the very low pK_a of the proton in *B* and the alkaline conditions of the reaction, proton loss from *B* will be extremely rapid. In contrast, loss of *p*-nitrophenolate is known from the isotope effect data to be the rate-limiting step in the overall mechanism. From these relative rate considerations, *B*−*D*−*E* is clearly the most likely reaction path. The formation of intermediate *D* will be rapid and essentially irreversible under the alkaline reaction conditions, and *D* will therefore partition completely forward by loss of *p*-nitrophenolate to give *E*. Under these circumstances, only isotope effects on the formation of

Scheme 2



D would be observed, and therefore, no ^{15}N isotope effect (^{15}k = 1) in the leaving group is expected since the bond to the leaving group has not broken. The experimentally observed magnitude of ^{15}k thus rules out this associative mechanism. An alternative direct route from A to D via concerted deprotonation and nucleophilic attack predicts a significant value for ^{D}k and a ^{15}k of unity and is therefore also ruled out by the experimental data.

Conclusion

Isotope effect studies have provided detailed mechanistic information for the hydrolysis of **2** by **1** that could not be obtained from basic kinetic studies. This work demonstrates the utility of secondary ^{15}N isotope effects for studying metal complex-promoted reactions of phosphate esters with a 4-nitrophenol leaving group. From the data reported above, **1**-catalyzed hydrolysis of activated phosphate diesters is shown to proceed through a concerted reaction mechanism. Further data for labile and inert metal complex-promoted hydrolysis of activated phosphate diesters will need to be collected to determine if the proposed mechanism holds true for other metal-promoted hydrolyses.

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Supporting Information Available: Tables of initial rates for the temperature-dependent hydrolysis of **2** by **1** and of calculated isotope effect values and an Arrhenius plot (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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