J|A|C|S
COMMUNICATIONS

Published on Web 09/27/2008

Enzyme-like Acceleration for the Hydrolysis of a DNA Model Promoted by a Dinuclear Zn(II) Catalyst in Dilute Aqueous Ethanol

C. Tony Liu, Alexei A. Neverov, and R. Stan Brown*

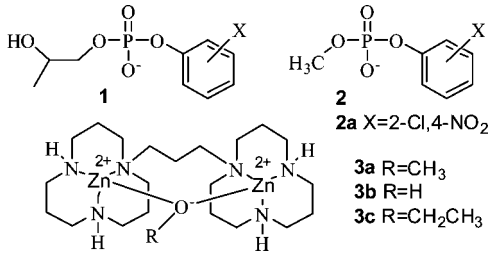
Department of Chemistry, Queen's University, Kingston, Ontario, Canada, K7L 3N6

Received July 24, 2008; E-mail: rsbrown@chem.queensu.ca

Molecules containing phosphate diester moieties $(RO)PO_2^-(OR')$ serve important biological functions because of their inherent stability toward hydrolytic and nucleophilic cleavage, making this functional group ideal for the preservation of genetic information in RNA and DNA.[1] Several enzymes that cleave RNA and DNA contain metal ions such as Zn^{2+},[2] and considerable research has been directed at understanding the mechanisms of hydrolysis and alcoholysis of RNA, DNA, and their model systems,[2,3] as well as toward designing M^{2+}-containing catalysts for facilitating their cleavage.[4]

We recently showed that the cleavages of the RNA and DNA models **1** and **2** promoted by the $Zn(II)_2$ complex **3**[5,6] in methanol and ethanol are accelerated by 10^{12} times or more relative to the background methanolysis reactions. In water, complex **3b** is no more active than the mono-Zn(II) complex of 1,5,9-triazacyclododecane toward the cleavage of phosphate diesters,[7] and we surmised that the acceleration of catalysis in methanol is attributable to a medium effect of reduced dielectric constant and polarity.[5,6] The catalyzed cleavages in methanol, although fast, are transesterifications and not hydrolytic reactions. In the case of the RNA models, the first formed product arises from intramolecular ring closure as it does in water, but in the DNA model cases the OAr leaving group is replaced by OR (Scheme 1).

Scheme 1



The X-ray structure of **3b** with a bridging HO^- was determined[5c] as grown from methanol solution at ambient conditions. The X-ray structure of the di-Cu(II) complex of the same ligand, also grown at ambient temperature, shows both a bridging hydroxide and a bridging water, the latter being replaced by a bridging phosphate when the crystal is grown in the presence of dibenzyl phosphate.[5d] This suggests that the catalyst might have a special affinity for a bridging HO^- ion, so under appropriate conditions the medium effect might be harnessed to accelerate the **3**-promoted hydrolytic reactions of phosphate diesters.[8] We report here a realization of this goal obtained through a study of the cleavage of the DNA model **2a** promoted by **3** in ethanol which reveals not only a large rate acceleration but an interesting phenomenon where as little as 3.8 vol % (2.1 M) of water in ethanol leads to 93:7 ratio of the hydrolysis product (**4**) to ethanolysis product (**5**).

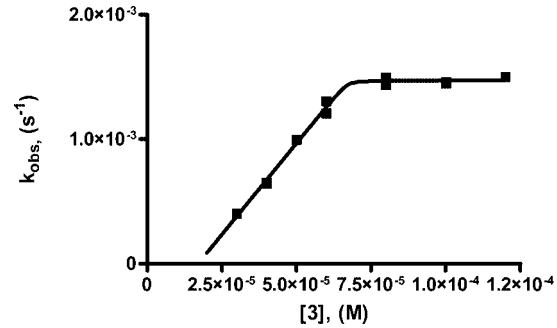


Figure 1. A plot of the observed first order rate constant for cleavage of **2a** (5×10^{-5} M) in ethanol with 28 mM H_2O vs varying [**3**], $T = 25$ °C, ${}^{s}_{s}pH = 7.90$.[10,11] The small apparent x-intercept of $\sim 1.7 \times 10^{-5}$ M is attributed to a dissociation of Zn^{2+} from **3** at very low concentrations.[5,6,9]

$$(CH_3O)PO_3^{2-} + (CH_3O)(CH_3CH_2O)PO_2^-$$
4 **5**

There are two main points of note. First, the plot in Figure 1 of the observed rate constant for cleavage of **2a** (5×10^{-5} M added as the acid) promoted by varying [**3**] with added equimolar NaOEt in anhydrous ethanol (used as supplied, but contains 28 mM H_2O by Karl Fischer titration) shows very strong **3** + **2a** saturation binding, followed by a chemical step liberating the phenol.[9] Nonlinear least-squares fitting of the data to a universal binding equation[5,6,9] gives the computed line through the data. The fit is based on an upper limit for the complex dissociation constant (K_d) of 3.2×10^{-8} M (determined iteratively by varying the K_d until the goodness of fit did not change[9]) and maximum catalytic rate constant for release of 2-Cl-4-NO_2-phenol (k_{cat}^{max}) of 1.47×10^{-3} s^{-1}. The apparent second order rate constant for the catalyzed reaction (k_2^{cat}), given as k_{cat}^{max}/K_d, is $\geq 4.6 \times 10^4$ M^{-1} s^{-1} which is a factor of at least 8.4×10^{10} larger than the second order rate constant (k_2^{EtO}) for the ethoxide promoted reaction of **2a** in ethanol $(5.5 \pm 0.3) \times 10^{-7}$ M^{-1} s^{-1}.[9]

Second, the products of cleavage of **2a** mediated by **3** in ethanol containing varying, but small, amounts of water (8 mM $\leq$ [H_2O] $\leq$ 2.1 M) were quantitatively determined by ^1H NMR analysis of the ethoxy, methoxy, and phenol peaks of the products isolated after reaction and confirmed by MS and ^{31}P NMR (see Table S2; sample NMR spectra are presented in Figures S1−S4[9]). In Figure 2 is a plot of the % hydrolytic product (**4**) as a function of added H_2O. To complement the above product determinations, the kinetics of the cleavage of 2.5 mM **2**a promoted by 2.5 mM **3** in ethanol containing 1 equiv of NaOEt were investigated at a few [H_2O]. The results, shown in Figure 3 (data tabulated in Table S7, Supporting Information), indicate a drop of slightly more than a

10.1021/ja805801j CCC: $40.75

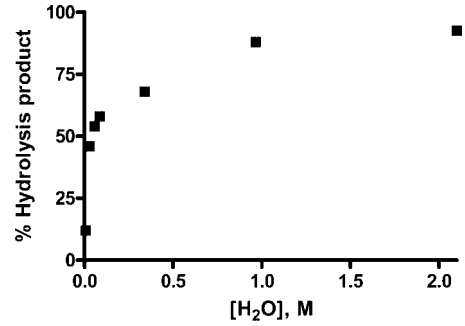


Figure 2. A plot of the percentage of analyzed hydrolysis product ($CH_3OPO_3^{2-}$) produced from the reaction of 2.5 mM **2a** promoted by 2.5 mM **3** in ethanol with varying amounts of water at room temperature.

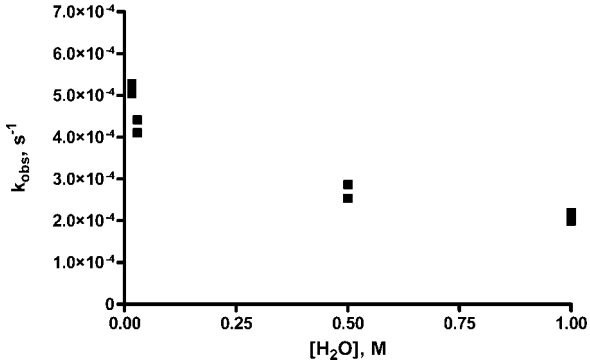


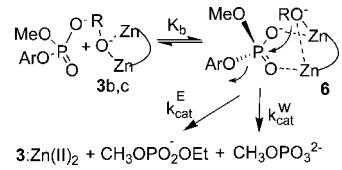
Figure 3. A plot of the observed first order rate constant for cleavage of **2a** (2.5 mM) in ethanol with 2.5 mM of **3** in ethanol vs varying [H_2O] in ethanol at $T = 25$ °C. The rate constants were determined from initial rate methods where the first 5−10% of the abs vs time trace for the appearance of the phenolic product at 323 nm was fitted to a standard linear regression model.

factor of 2 in passing from 16 mM to 1 M water, probably due to a change in the medium since it was previously determined that complex **3** has an unremarkable reactivity in an aqueous solution.[7] One also sees that the rate constant for the catalyzed reaction at the 25-times higher concentration of catalyst is about three times lower than under the conditions of Figure 1, probably due to the inhibitory effect of triflate counterions.[5,6]

The unusual points brought to light in this study are the large amount of hydrolysis and increase in rate of the hydrolytic rates brought about by the combination of a dinuclear Zn(II) catalyst and a medium effect in ethanol. That as little as 28 mM of water (0.05 vol%), in the presence of an overwhelming excess of ethanol gives 46% of hydrolysis product from a relatively inert phosphodiester suggests a process that selects for H_2O or $^-$OH (either external or di-Zn(II) complex-coordinated) relative to ethanol or ethoxide as the active nucleophile attacking the **3**:**2a** complex. Solvolytic reactions in mixed ethanol/water media are known to be complex[12] but the available evidence allows us to rule out external H_2O and $^-$OH as being responsible for the hydrolysis, leaving complex **3b** with an intramolecularly coordinated HO^- as the most likely catalyst.

Our earlier study of the **3**-catalyzed methanolysis[5b] of methyl *p*-nitrophenyl phosphate indicated that the rate maximized at a 1:2:1 ratio of ligand/Zn^{2+}/$^-OCH_3$, suggesting that the optimally effective catalyst is **3a**. The same is seen for the **3**-catalyzed reaction of **2a** in ethanol[9,6b] indicating that the transition state contains a 1:2:1:1 ratio of ligand, Zn(II), phosphate, and lyoxide (EtO^- and/or HO^- or its chemical equivalent). This assertion requires that the species leading to the hydrolysis product is a complex-coordinated HO^-,

Scheme 2. Charges Omitted for Clarity



so we must rule out reasonable alternatives such as attack of hydroxide on the **3**:**2a** complex. On first inspection, one might expect, on the basis of autoprotolysis constants, that water ($K_W = 10^{-14}$) is more acidic than ethanol ($K_E = 10^{-19.1}$).[11] In mixed solvents, this ignores the medium effect of transferring the dissociating water into a less polar medium so it is expected to be less dissociated in ethanol than in water. However, the exact calculation of the effects requires knowledge of how the various equilibria and activity coefficients depend on the mixed solvent which is not known at the present time.[13]

Fortunately, we can experimentally estimate the ionization constant of water in ethanol. In a little known, but important paper, Caldin and Long[14] presented evidence that in ethanol, or ethanol containing "not more than a few percent water", the acid dissociation constants of ethanol and water are similar with water being slightly weaker. We confirmed this by determining the rate constants and cleavage products of cleavage of *p*-nitrophenyl benzoate under basic conditions in ethanol containing 0.53 and 1.03 M water where the amounts of hydrolysis products are 1.0 and 2.3% (Tables S2−S5, Supporting Information[9]). Using a computational approach based on that provided,[14] we find that the ratio between the acid dissociation constant of water and ethanol ($^s_sK_a^W/^s_sK_a^E$)[9] in ethanol at these two water concentrations is 0.83 and 0.91. Assuming a linear correlation of the ratio with [water], $^s_sK_a^W/^s_sK_a^E = 0.75$ was computed for ethanol containing 28 mM water, showing water to be slightly less acidic than ethanol at the concentrations employed, in agreement with what Caldin and Long determined.[14] The acid dissociation constant of ethanol containing 28 mM H_2O is assumed to be reliably computed from the autoprotolysis constant of pure ethanol as $^s_spK_a^E = -\log(10^{-19.1}/[EtOH]) = 20.33$ while that of water ($^s_spK_a^W$) in the same medium is 20.45.

The so-determined acidities of water and ethanol indicate that free $^-$OH cannot be the active nucleophile in hydrolyzing **3**:**2a** in ethanol under the kinetic conditions of Figure 1. The exact treatment is shown in section 2a of the Supporting Information, but is summarized as follows. At $^s_spH = 7.9$ and $[H_2O] = 0.028$ M, $[OH^-] = {}^s_sK_a^W ([H_2O]/[H^+]) = 7.9 \times 10^{-15}$ M. To account for the fact that 46% of the reaction at this water content gives hydrolysis product, the rate constant for external $^-$OH attack would be $(0.46 \times k_{cat}^{max})/[HO^-] = 8.6 \times 10^{10}$ M^{-1} s^{-1} which exceeds the diffusion limit in ethanol by a factor of 8.6.[15] By exclusion, the active species is most simply formulated as **3b**, or some closely related complex having an asymmetrical doubly coordinated or singly coordinated HO^-.

Given in Scheme 2 is a proposed pathway which is common to **3b** and **3c** catalyzed cleavage of **2a**. The scheme builds on our previous interpretation that the substrate becomes doubly activated through binding to both Zn(II) ions followed by an intramolecular delivery of the coordinated nucleophile (ethoxide or hydroxide). Structure **6** in the scheme is proposed by analogy to the X-ray structure of the di Cu(II) complex where a bridging dibenzyl phosphate and a bridging hydroxide are observed to complete the five-coordinate metal ion ligation.[5d] Whether the actual attack is stepwise, through a phosphorane intermediate, or concerted cannot be established with the information at hand.[3,6a]

It has been stated that "the active sites of enzymes are non-aqueous, and the effective dielectric constants resemble those in organic solvents rather than that in water".[16] The low dielectric interior of enzymes also means that ion−dipole and ion−ion interactions will be stronger than in water[17] and thus might provide a very effective way to lower the transition state energies for metal promoted reactions of anionic substrates. However, it is difficult to quantify the effect given the complexity of the enzyme catalyzed processes. The present data indicate that the reduced dielectric constant of ethanol relative to water (24.3 vs 78) plays an important role in achieving the acceleration for the hydrolytic process observed here with a rather simple dinuclear Zn(II) complex. Just how great is the acceleration can be quantified in two simple ways,[9] comparing the second order rate constants (k_{cat}^{max}/K_d) for the catalytic reaction and those for the lyoxide reactions or by comparing the k_{cat}^{max} value for cleavage of the **3**:**2a** complex to the background lyoxide reactions at ${}^{s}_{s}pH = 7.90$. For the first method, the 54:46 ratio of the products **5**:**4** at 28 mM H_2O, requires k_2^{cat} (ethanolysis) $= 0.54 \times (k_{cat}^{max}/K_d) = 2.48 \times 10^4\ M^{-1}\ s^{-1}$ and k_2^{cat} (hydrolysis) $= 2.11 \times 10^4\ M^{-1}\ s^{-1}$. Initial rate experiments indicate that the rate constant for the lyoxide reaction does not increase with increasing $[H_2O]$, so that an upper limit for the k_2 value for the hydroxide reaction is approximately that of the ethoxide reaction in the absence of catalyst (see Supporting Information). Thus, the accelerations are k_2^{cat} (ethanolysis)$/k_{EtO^-} = 4.5 \times 10^{10}$, and k_2^{cat} (hydrolysis)$/k_{HO^-} \geq 3.8 \times 10^{10}$.

When comparing the catalytic rate accelerations relative to the assumed base-promoted background reactions at ${}^{s}_{s}pH = 7.90$ in ethanol with 28 mM water, the ${}^{s}_{s}K_a^{w}$ and ${}^{s}_{s}K_a^{E}$ values indicate that $[OH^-] = 7.9 \times 10^{-15}$ M and $[EtO^-] = 6.3 \times 10^{-12}$ M.[9] Thus $k_{obs}^{EtO} = 3.5 \times 10^{-18}\ s^{-1}$ while the upper limit for $k_{obs}^{HO} = 4.4 \times 10^{-21}\ s^{-1}$. Since 54% of the k_{cat}^{max} term of $1.47 \times 10^{-3}\ s^{-1}$ leads to ethanolysis product, the acceleration for this process is $(0.54 \times k_{cat}^{max})/[EtO^-] \leq 2.3 \times 10^{14}$ and the hydrolysis is accelerated by $\geq 1.6 \times 10^{17}$, suggesting that complex **3** promotes the hydrolytic reaction at least 1000 times more efficiently than ethanolysis.

Phosphodiesterases are among the most efficient enzymes in promoting hydrolytic reactions relative to their background reactions, with accelerations of $\sim 10^{17}$ being reported.[18] Despite active investigation of numerous simple metal ion containing catalytic systems for cleaving phosphodiesters, none of those reported to date demonstrate a catalytic acceleration for hydrolysis approaching that of the enzymes. In this study, a model system comprising a dinuclear Zn(II) complex and a synergistic medium effect provided by ethanol containing small amounts of H_2O gives an impressive acceleration for the hydrolysis of a phosphodiester (a factor of 1.6×10^{17} relative to the background HO^--promoted reaction). Catalyst **3** shows a very large selectivity for activating water as a nucleophile in the presence of an overwhelming concentration of ethanol. These results demonstrate in a convincing way an underappreciated mode by which very large rate accelerations for hydrolytic reactions might be achieved by the coupling of catalytically important functional groups and medium effects.

Acknowledgment. The authors acknowledge financial assistance of NSERC, the Canada Council for the Arts through the award of a Killam Research fellowship to RSB, and Queen's University. In addition they thank Prof. E. Bosch, Prof. M Rosés, and Prof. J. P Guthrie for helpful discussions and the referees of the initial manuscript for their constructive criticisms. C.T.L. thanks NSERC for a PGSD Scholarship.

Supporting Information Available: Experimental details, supporting text, Figures S1−S6, Tables S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Weston, J. *Chem. Rev.* **2005**, *105*, 2151.
(2) Sträter, N.; Lipscomb, W. N.; Klabunde, T.; Krebs, B. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2024. Wilcox, D. E. *Chem. Rev.* **1996**, *96*, 2435.
(3) Perrault, D. M.; Anslyn, E. V. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 432.
(4) (a) Mancin, F.; Tecillia, P. *New J. Chem.* **2007**, *31*, 800. (b) Molenveld, P.; Engbertsen, J. F. J.; Reinhoudt, D. N. *Chem. Soc. Rev.* **2000**, *29*, 75. (c) Williams, N. H.; Takasaki, B.; Wall, M.; Chin, J. *Acc. Chem. Res.* **1999**, *32*, 485. (d) Morrow, J. R.; Iranzo, O. *Curr. Opin. Chem. Biol.* **2004**, *8*, 192.
(5) (a) Brown, R. S.; Neverov, A. A. *Adv. Phys. Org. Chem.* **2007**, *42*, 271. (b) Neverov, A. A.; Lu, Z.-L.; Maxwell, C. I.; Mohamed, M. F.; White, C. J.; Tsang, J. S. W.; Brown, R. S. *J. Am. Chem. Soc.* **2006**, *128*, 16398. (c) Bunn, S. E.; Liu, C. T.; Lu, Z.-L.; Neverov, A. A.; Brown, R. S. *J. Am. Chem. Soc.* **2007**, *129*, 16238. (d) Lu, Z.-L.; Liu, C. T.; Neverov, A. A.; Brown, R. S. *J. Am. Chem. Soc.* **2007**, *129*, 11642.
(6) (a) Neverov, A. A.; Liu, C. T.; Bunn, S. E.; Edwards, D.; White, C. J.; Melnychuk, S. A.; Brown, R. S. *J. Am. Chem. Soc.* **2008**, *130*, 6639. (b) Liu, C. T.; Neverov, A. A.; Brown, R. S. *J. Am. Chem. Soc*. Unpublished work.
(7) Kim, J.; Lim, H. *Bull. Korean Chem. Soc.* **1999**, *20*, 491.
(8) There is a report that some Cu(II) complexes with functionalized bipyridyl ligands give hydrolysis products in 95% ethanol/water but medium effects were specifically excluded as being operative. Kövári, E.; Krämer, R. *J. Am. Chem. Soc.* **1996**, *118*, 12704.
(9) See Supporting Information
(10) The symbol ${}^{s}_{s}pH$ is used for designation of pH in nonaqueous solvents; the autoprotolysis constant of ethanol is $10^{-19.1}$ so neutral ${}^{s}_{s}pH$ is 9.55.[11]
(11) Gibson, G. T. T.; Mohamed, M. F.; Neverov, A. A.; Brown, R. S. *Inorg. Chem.* **2006**, *45*, 7891.
(12) Ta-Shma, R.; Rappoport, Z. *Adv. Phys. Org. Chem.* **1992**, *27*, 239. Minegishi, S.; Kobayashi, S.; Mayr, H. *J. Am. Chem. Soc.* **2004**, *126*, 5174.
(13) Fonrodona, G.; Ràfols, C.; Bosch, E.; Rosés, M. *Anal. Chim. Acta* **1996**, *335*, 291.
(14) Caldin, E. F.; Long, G. *Nature* **1954**, 3757.
(15) Schwarz, H. A.; Gill, P. A. *J. Phys. Chem.* **1977**, *81*, 22.
(16) Cleland, W. W.; Frey, P. A.; Gerlt, J. A. *J. Biol. Chem.* **1998**, *273*, 25529.
(17) Richard, J. P.; Ames, T. L. *Bioorg. Chem.* **2004**, *32*, 354.
(18) Schroeder, G. K.; Lad, C.; Wyman, P.; Williams, N. H.; Wolfenden, R. *Proc. Nat. Acad. Sci. U.S.A.* **2006**, *103*, 4052.

JA805801J