Generality of Solvation Effects on the Hydrolysis Rates of Phosphate Monoesters and Their Possible Relevance to Enzymatic Catalysis

Piotr K. Grzyska,† Przemyslaw G. Czyryca,† Justin Golightly, Kelly Small, Paul Larsen, Richard H. Hoff,‡ and Alvan C. Hengge*

Utah State University, Department of Chemistry and Biochemistry, Logan, Utah 84322-0300

hengge@cc.usu.edu

Received September 10, 2001

Previous work by Kirby and co-workers revealed a significant acceleration of the rate of hydrolysis of *p*-nitrophenyl phosphate by added dipolar solvents such as DMSO. Activation parameters and kinetic isotope effects have been measured to ascertain the origin of this effect. The generality of this phenomenon was examined with a series of esters with more basic leaving groups. Computational analyses of the effects of desolvation of dianionic phosphate monoesters were carried out, and the possible effect of the transfer from water to the active site of alkaline phosphatase was modeled. The results are consistent with a desolvation-induced weakening of the P−O ester bond in the ground state. Other aryl phosphate esters show similar rate accelerations at high fractions of DMSO, but phenyl and methyl phosphates do not, and their hydrolysis reactions are actually slowed by these conditions.

Introduction

Phosphoryl transfer reactions are ubiquitous in biological systems where the formation and hydrolysis of phosphate monoesters comprises an essential regulatory mechanism for a host of cellular processes. For most phosphatases, the dianion of the phosphate monoester is the substrate for catalysis. Most of what is known about the chemistry of phosphate monoesters has come from the study of aryl phosphates. The uncatalyzed hydrolysis reactions of the dianions of aryl phosphate monoesters proceed through a concerted mechanism with a very loose transition state (Figure 1) in which the phosphoryl group resembles metaphosphate ion, bond cleavage to the leaving group is extensive, and bond formation to the nucleophile is minimal. The extensive evidence for this mechanism has been reviewed.[1,2] While bond formation to the nucleophile is not far advanced in the transition state, data from linear free-energy relationships indicate that metaphosphate is not an intermediate,[3] and results with phosphates made chiral by the use of oxygen isotopes show that the bond is sufficiently far developed to effect inversion except when the phosphoryl acceptor is the sterically hindered *tert*-butyl alcohol.[4,5]

In 1986 Kirby and Abell reported that added DMSO on the hydrolysis of *p*-nitrophenyl phosphate (pNPP) dianion resulted in up to a 10^6-fold rate acceleration when the level of DMSO reached 95%.[6] It was speculated that the faster rate arose from disruption of hydrogen bonding to the phosphoryl group, resulting in a weakening of the P−O ester bond, and that a similar effect might arise from binding to a phosphatase active site and contribute to enzymatic catalysis. In the uncatalyzed reaction, the negative charges on the phosphoryl group assist in expulsion of the leaving group. Thus, it is a logical hypothesis that desolvation of the phosphoryl group would destabilize the reactant by making these charges more available to assist in expulsion of the leaving group. However, there are alternative explanations for the rate acceleration imparted by DMSO. Reactions involving nucleophilic attack are often faster in aprotic solvents because the nucleophile is less solvated. Thus, under these conditions the mechanism may change to one with significant nucleophilic involvement by either water or hydroxide.

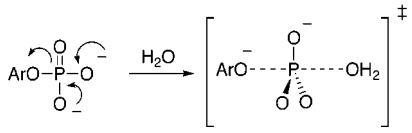


Figure 1. Loose transition state for hydrolysis reactions of aryl phosphate ester dianions.

We have carried out experiments to better understand the nature of the rate acceleration imparted by added DMSO to aqueous solutions of pNPP. To ascertain whether rate accelerations occur for phosphate monoesters having less acidic leaving groups, the effect of DMSO on the hydrolysis rates of several aryl phosphates, phenyl phosphate, and methyl phosphate have been examined.

* Corresponding author. Fax: 435-797-3390.
† These authors contributed equally to this work.
‡ Present address: Associate Professor, Department of Chemistry, U.S. Military Academy, West Point.

(1) Hengge, A. C. In *Comprehensive Biological Catalysis: A Mechanistic Reference*; Sinnott, M., Ed.; Academic Press: San Diego, CA, 1998; Vol. 1, pp 517−542.
(2) Thatcher, G. R. J.; Kluger, R. *Adv. Phys. Org. Chem.* **1989**, *25*, 99−265.
(3) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7579−7586.
(4) Friedman, J. M.; Freeman, S.; Knowles, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 1268−1275.
(5) Buchwald, S. L.; Friedman, J. M.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 4911−4916.
(6) Abell, K. W. Y.; Kirby, A. J. *Tetrahedron. Lett.* **1986**, *27*, 1085−1088.

10.1021/jo016104p CCC: $22.00
Published on Web 01/17/2002

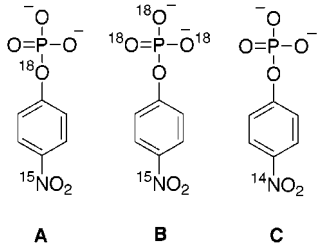


Figure 2. Isotopic isomers used for measurement of the bridge (A and C) and nonbridge (B and C) ^{18}O isotope effects.

Experimental Section

The disodium salts of *p*-nitrophenyl phosphate and of phenyl phosphate were commercial products and were recrystallized before use. The bis(cyclohexylammonium) salts of *p*-chlorophenyl phosphate, *m*-bromophenyl phosphate, and *p*-cyanophenyl phosphate were prepared by the method of Bourne and Williams.[7] These were converted to their sodium salts by a cation exchange column using SP-C25 resin equilibrated with sodium acetate. The esters were pure by proton and ^{31}P NMR (see Supporting Information).

Methyl phosphate was synthesized by a modification of the method described by Kirby.[8] In a 50 mL round-bottom flask with condenser placed in an ice water bath, 0.010 mol of dry phosphorous acid was dissolved in 20 mL of methanol to which solution 0.035 mol of triethylamine was added. Then, 0.015 mol of solid iodine was added very slowly in such a manner that rapid evolution of heat was avoided. The cold bath was removed, and the reaction was allowed to warm to room temperature. After approximately 15 min, the reaction mixture was poured into about 200 mL of acetone with 5 g of ground NaOH as a suspension. A cloudy suspension of the disodium salt of methyl phosphate was filtered off. The product was recrystallized from methanol to yield methyl phosphate in 80% yield as the disodium salt. The product was pure by ^{1}H and ^{31}P NMR.

The isotopic isomers of *p*-nitrophenyl phosphate used for measurement of kinetic isotope effects are shown in Figure 2. Natural abundance *p*-nitrophenyl phosphate, [^{14}N]-*p*-nitrophenyl phosphate, [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate, [^{14}N]-*p*-nitrophenol, and [^{15}N, ^{18}O]-*p*-nitrophenol were synthesized as described previously.[9] [^{14}N]-*p*-nitrophenol and [^{15}N, ^{18}O]-*p*-nitrophenol were then mixed to reconstitute the natural abundance of ^{15}N, and then the mixture was phosphorylated to produce *p*-nitrophenyl phosphate using the method cited above. This mixture of isotopic isomers (A and C, Figure 2) was used for determination of $^{18}k_{bridge}$. Isomers B and C were also mixed to reconstitute the natural abundance of ^{15}N and used for determination of $^{18}k_{nonbridge}$. The isotopic abundance of the mixtures was determined by isotope ratio mass spectrometry. Scheme 1 summarizes the positions in the pNPP reactant and the nomenclature for the isotope effects measured.

Isotope Effect Determinations. Isotope effects were measured by isotope ratio mass spectrometry. The N^{15} isotope effects were measured using the natural abundance compound. The O^{18} isotope effects were measured by the remote label method[10] using the nitrogen atom as a reporter for isotopic fractionation at the bridge or nonbridge oxygen atoms.[9]

Isotope effects were measured at 85 °C in 90% DMSO as follows. The disodium salt (30 mg) of the appropriate isotopically labeled form of the substrate was dissolved in 500 μL of 1 N NaOH at room temperature. This was added to 19.5 mL of 92.3% DMSO/H_2O at 85 °C. After partial hydrolysis, the reactions were stopped by pouring the mixture into 200 mL of ice-cold water. An aliquot was assayed for *p*-nitrophenol in basic solution at 400 nm; a second aliquot was added to TRIS buffer at pH 9 and treated with alkaline phosphatase to completely hydrolyze the remaining reactant and then assayed similarly. The fraction of reaction was determined from these two measurements. The remainder of the reaction mixture was titrated to pH 5 and extracted with diethyl ether three times to extract the product *p*-nitrophenol. These ether layers were dried over magnesium sulfate, and DMSO was removed by passing the ether solution through a column of silica gel (320−400 mesh). The resulting ether solution was evaporated to dryness, and the *p*-nitrophenol was further purified by sublimation before isotopic analysis by isotope ratio mass spectrometry using an ANCA-NT combustion system in tandem with a Europa 20−20 isotope ratio mass spectrometer.

The aqueous layer, containing the unreacted pNPP, was made alkaline and, water was removed at 35−40 °C by rotary evaporation. By the time most of the water had been removed and the reactant mixture had once again achieved a high proportion of DMSO, complete hydrolysis of the residual pNPP was rapid. After >10 half-lives, this mixture was added to 200 mL of water, titrated to pH 5, and treated as described above to recover *p*-nitrophenol.

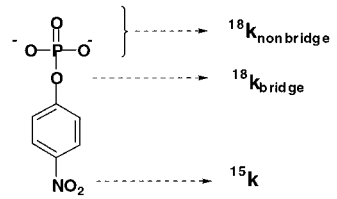
Isotope effects were calculated from the isotopic ratios at partial reaction in the *p*-nitrophenol product (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equations 1 and 2 were used to calculate the observed isotope effect either from R_p and R_o or from R_s and R_o, respectively, at the fraction of reaction f.[11] Thus, each experiment yields two independent determinations of the isotope effect.

$$\text{isotope effect} = \log(1 - f)/\log(1 - f(R_p/R_o)) \quad (1)$$

$$\text{isotope effect} = \log(1 - f)/\log[(1 - f)(R_s/R_o)] \quad (2)$$

R_o was determined by two methods: from unreacted pNPS by isotope ratio mass spectrometry, and, as a control, from *p*-nitrophenol isolated after complete hydrolysis of a sample

Scheme 1. Diagram Showing the Position and Nomenclature of the Isotope Effects Measured, and the Isotope Effects for Hydrolysis in Aqueous Solution and in 90% DMSO[a]



Isotope effect	Aqueous reaction, 95 °C [9]	90% DMSO, 85 °C
$^{18}k_{nonbridge}$	0.9994 (5)	1.0000 (3)
$^{18}k_{bridge}$	1.0202 (5)	1.0159 (5)
^{15}k	1.0028 (2)	1.0019 (2)

[a] Standard errors in the last decimal place are in parentheses.

(7) Bourne, N.; Williams, A. *J. Org. Chem.* **1984**, *49*, 1200−1204.
(8) Kirby, A. J. *Chem. Ind.* **1963**, 1877−1878.
(9) Hengge, A. C.; Edens, W. A.; Elsing, H. *J. Am. Chem. Soc.* **1994**, *116*, 5045−5049.
(10) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300−3306.
(11) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15−76.

of pNPS using the same isolation and purification procedures used in the isotope effect experiments. The agreement of these two numbers demonstrated that, within the experimental error, no isotopic fractionation occurs as a result of the procedures used to isolate and purify the *p*-nitrophenol.

Kinetics of Aryl Phosphates and Methyl Phosphate. The hydrolysis of the dianion of *p*-nitrophenyl phosphate in 95% DMSO in the presence of 20 mM tetrabutylammonium hydroxide, with ionic strength maintained using tetrabutylammonium bromide, was examined as described by Kirby et al.[6] The reaction rate was monitored to completion by following the release of *p*-nitrophenolate at 400 nm at 283, 297, 303, and 312 K.

For determination of the effect of the leaving group on the rate, the hydrolysis rates of *p*-chlorophenyl phosphate, *m*-bromophenyl phosphate, and *p*-cyanophenyl phosphate in 95% DMSO were measured using the initial rate method. The phosphate ester concentration was 2.5 mM for *p*-chlorophenyl phosphate and for *m*-bromophenyl phosphate and 1.25 mM for *p*-cyanophenyl phosphate. Reaction mixtures were 20 mM in [$^-$OH]. The dependence of the rates on hydroxide concentration rates were examined for [$^-$OH] between 20 and 40 mM, keeping ionic strength constant using tetrabutylammonium bromide. The rates were followed by periodically adding aliquots of the reaction mixture to 0.1 N NaOH and measuring the absorbance of the liberated phenolate ion at 310, 295, and 295 nm for *p*-chlorophenyl phosphate, *m*-bromophenyl phosphate, and *p*-cyanophenyl phosphate, respectively. Endpoints for these reactions were obtained by adding an aliquot of the reaction mixture to a solution of *E. coli* alkaline phosphatase in 100 mM TRIS buffer at pH 9, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$ and assaying for liberated phenol after 24 h.

Because of the very small change in UV absorption that accompanies hydrolysis of phenyl phosphate and methyl phosphate, ^{31}P NMR was used to monitor the hydrolysis rates of these two esters. The reactions were carried out in sealed quartz tubes as described by Wolfenden.[12] Tubes containing from 200 to 450 μL of the reaction solution were placed in a thermostated silicone oil bath. After appropriate times, samples were removed and cooled, and the contents were diluted with D_2O. For the aqueous hydrolysis of phenyl and methyl phosphates, reactions were carried out at pH 11 in 0.3 M carbonate buffer with an initial reactant concentration of 50 mM. The experiments with phenyl and methyl phosphates in a DMSO/water mixture required a lower fraction of DMSO (80%) in order to solubilize a sufficient concentration (5 mM) of the reactant to allow accurate monitoring by NMR. The concentration of tetrabutylammonium hydroxide was varied from 30 to 60 mM, with ionic strength maintained at 75 mM using tetrabutylammonium bromide. After appropriate times, samples were removed and cooled, and the contents were diluted with D_2O and analyzed by NMR.

Computational Analyses. The effect of solvation on the scissile P−O ester bond of *p*-nitrophenyl, phenyl, and methyl phosphates was examined computationally using the Gaussian 98 revision 7 package on the HF/6-31++G** level.[13] The structures of these three phosphate esters were optimized in the gas phase, in the gas phase with one molecule of water explicitly modeled, and using the polarizable continuum models (PCM) for DMSO and for water.

Table 1. Phosphorus−Oxygen Ester Bond Lengths (Å) for Methyl, Phenyl, and *p*-Nitrophenyl Phosphate Dianions from Calculations at the HF/6-31++G Level, Using the PCM Method as Implemented in Gaussian 98.[a]**

	Leaving Group/pK_a		
	−OMe/ 15.5	−OPh/ 9.95	−OpNPh/ 7.14
gas phase, HF/6-31++G**	1.730	1.825	1.989
1 molecule of H_2O *explicitly*, HF/6-31++G**	1.709	1.785	1.866
DMSO, HF/6-31++G**, PCM	1.680	1.725	1.866
water, HF/6-31++G**, PCM	1.663	1.699	1.729
ONIOM HF/6-31++G**:PM3 **high level**: methyl phosphate; **low level**: seven solvating molecules of water	1.678		
same system as above, fully in HF/6-31++G**	1.674		
ONIOM HF/6-31++G**:PM3 **high level**: methyl phosphate and two Zn^{2+} ions; **low level**: residues of alkaline phosphatase active site	1.707		

[a] z-Matrix for each final structure is available in Supporting Information

Additional calculations were carried out with the methyl phosphate dianion (the other esters were excluded for practical considerations). These calculations involved an evaluation of the applicability of the ONIOM[1] method[14] to study the influence of the environment on the P−O bond length using a test structure of methyl phosphate solvated with seven explicitly introduced molecules of water and a calculation of the geometry of the alkaline phosphatase−substrate complex. In the former, methyl phosphate solvated by seven water molecules was examined fully at the HF/6-31++G** level, and then for comparison, using the ONIOM method (the ONIOM method allows the model to be split into a number of layers of different levels of theory and thus allows for reduction of the computational time and memory usage) with the HF/6-31++G** layer used for the phosphate and PM3 for the water molecules. Both approaches yielded basically the same results (Table 1), which validated the applicability of the ONIOM method. Then, the ONIOM method was used for optimization of methyl phosphate bound to the active site of alkaline phosphatase. A fragment of the ALK1[15] structure, corresponding to the active site, was used as an initial structure (Figure 3). Free-peptide linkages were terminated as amides, and the inorganic phosphate in ALK1 was substituted with methyl phosphate in its dianionic form prior to optimization. To preserve the proper geometry of the active site, a number of atoms of the structure were set as nonoptimizable.

Results

To compare these results with prior data reported with pNPP in 95% DMSO, and to maximize the expected rate acceleration imparted by the dipolar aprotic solvent, experiments were conducted in 95% DMSO whenever possible. Some experiments required the use of lower DMSO fractions as discussed above. Table 2 shows the percentages of DMSO that were used in particular experiments.

Aryl Phosphate Esters. The hydrolysis rate of *p*-nitrophenyl phosphate dianion in 95% DMSO and the independence of the rate from the concentration of hydroxide agree with results previously reported by Kirby et al.[6] The rate of hydrolysis was measured over the temperature range from 10 to 39.5 °C, and an Eyring plot

(12) Wolfenden, R.; Ridgway, C.; Young, G. *J. Am. Chem. Soc.* **1998**, *120*, 833−834.

(13) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, revision 7.0; Gaussian, Inc.: Pittsburgh, PA, 1998.

(14) Dapprich, S.; Komaromi, I.; Byun, K. S.; Morokuma, K.; Frisch, M. J. *J. Mol. Struct.: THEOCHEM* **1999**, 1−21.

(15) Kim, E. E.; Wyckoff, H. W. *J. Mol. Biol.* **1991**, *218*, 449−464.

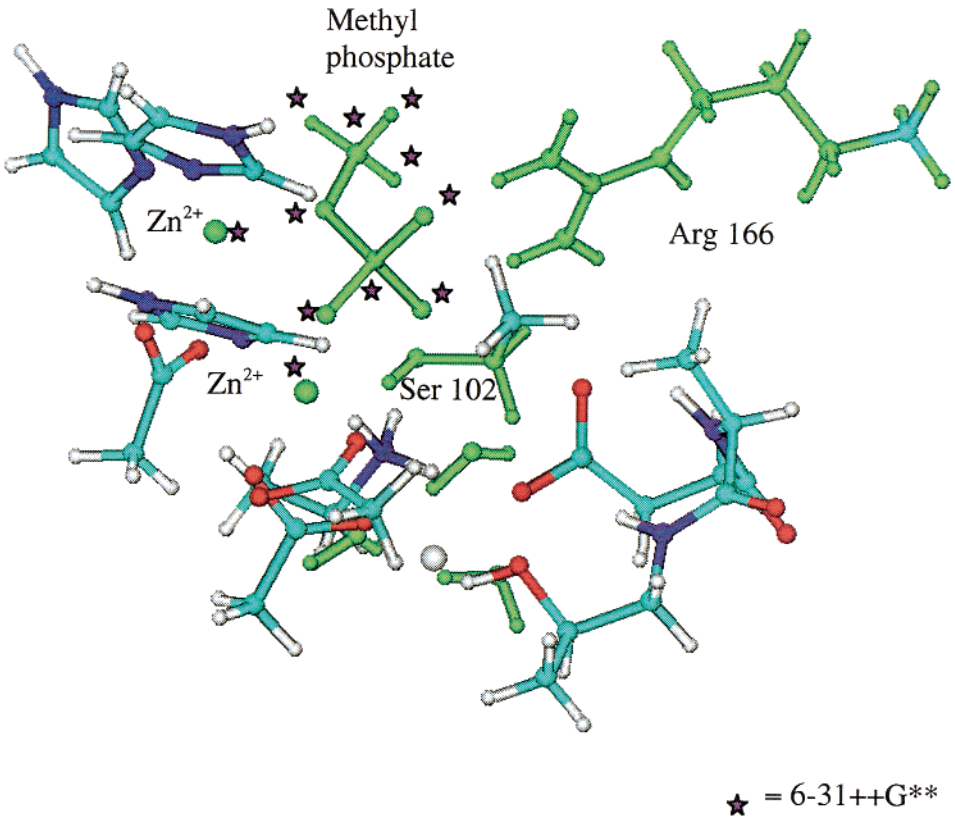


Figure 3. Model of the active site of ALK1. Optimizable region of the active site is shown in green. Atoms belonging to a high, ab initio level for the ONIOM method are marked with stars; all other atoms were modeled at the PM3 level.

Table 2. Summary of Percentages of DMSO Used in Experiments with Phosphate Monoester Dianions

Rate measurements and measurements of Brønsted β_{lg} with *p*−nitrophenyl phosphate, *p*−chlorophenyl phosphate, *m*−bromophenyl phosphate, and *p*−cyanophenyl phosphate	95% DMSO
Rate measurements with phenyl phosphate and methyl phosphate	80% DMSO
Kinetic isotope effects with *p*-nitrophenyl phosphate	90% DMSO

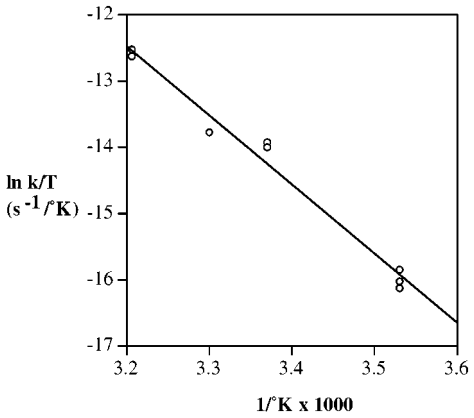


Figure 4. Eyring plot for the hydrolysis of pNPP dianion in 95:5 DMSO/water.

Table 3. Activation Parameters for Hydrolysis of the pNPP Dianion in Water and in 95% DMSO/Water[a]

	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (eu)
H_2O	30.6	+ 3.5
95:5 DMSO/H_2O	20.7 ± 0.6	−5.7 ± 0.6

[a] Aqueous values are from ref 17.

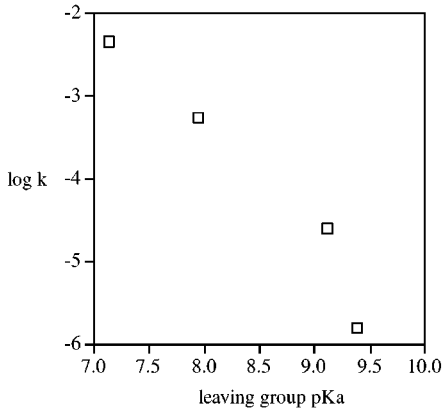


Figure 5. Brønsted plot showing the log of the pseudo-first-order rate constant (in sec^{-1}) versus leaving group pK_a for the hydrolysis of *p*-chlorophenyl phosphate, *m*-bromophenyl phosphate, *p*-nitrophenyl phosphate, and *p*-cyanophenyl phosphate in 95% DMSO.

(Figure 4) was used to obtain the activation parameters $\Delta H^{\ddagger} = 20.7 \pm 0.6$ kcal/mol and $\Delta S^{\ddagger}_{39} = -5.7 \pm 0.6$ entropy units (eu) (Table 3).

The rate constants for the hydrolysis in 95% DMSO of *p*-nitrophenyl phosphate, of *p*-chlorophenyl phosphate, of *m*-bromophenyl phosphate, and of *p*-cyanophenyl phosphate were used to construct the Brønsted plot in Figure 5. If a line is fitted to all four points, a Brønsted slope of -1.4 ± 0.2 is obtained, with $r^2 = 0.948$. The phosphate ester having the leaving group with the highest pK_a, *p*-chlorophenol, undergoes reaction somewhat slower than predicted by a line passing through the points for the first three compounds. A fit to the first three points alone gives a Brønsted slope of -1.15 ± 0.01, with $r^2 =$

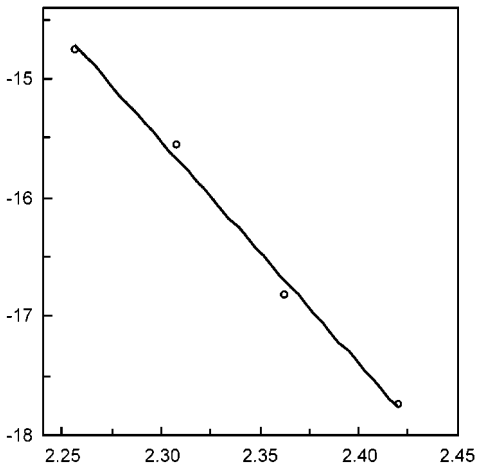


Figure 6. Eyring plot for hydrolysis of the phenyl phosphate dianion in aqueous solution, pH 11.

0.999. For comparison, the value obtained for the aqueous hydrolysis of a series of aryl phosphate dianions is −1.23.[16]

Phenyl and Methyl Phosphate Esters. NMR was used to follow the rates of the reactions of phenyl and methyl phosphate in aqueous and in 80% DMSO mixtures, and elevated temperatures were necessary to observe measurable rates of hydrolysis.

Analyses of the reaction progress of the aqueous hydrolysis of phenyl phosphate and methyl phosphate by ^{31}P NMR showed that both reactions followed simple first-order kinetics to completion. The measured rate for the aqueous hydrolysis of methyl phosphate matched that reported previously by Wolfenden et al.[12] The rate of hydrolysis of the phenyl phosphate dianion at pH 11, which is in the pH-independent range, was measured at temperatures of 140, 150, 160, and 170 °C. These data were used to construct the Eyring plot shown in Figure 6, which yielded a value for the enthalpy of activation of 37 ± 1 kcal/mol. The value for the entropy of activation at 39 °C was calculated for comparison with the reported value of +3.5 eu for pNPP at the same temperature.[17] The value for $\Delta S^{\ddagger}_{39}$ was obtained by using the extrapolated rate constant at 39 °C to calculate $\Delta G^{\ddagger}$ and then subtracting $\Delta H^{\ddagger}$ to obtain $T\Delta S$. This yields a value for $\Delta S^{\ddagger}_{39} = +7.3 \pm 0.6$ eu.

The aqueous reactions were well behaved, but the 80% DMSO reactions were accompanied by the gradual formation of brown-colored material and the gradual loss of the ^{31}P NMR signal. The NMR signal could be partially restored by treating the sample with EDTA; thus, it is possible that metals were being leached out of the quartz tubes by the solution. While neither of these reactions in DMSO/water could be followed to sufficient turnover for calculation of a rate constant, under some conditions the formation of product could be detected before the NMR signal was lost. The reactions were examined across a range of hydroxide concentrations in order to ensure that the reaction of the dianion was being observed (in aqueous solution, the monoanionic species undergoes hydrolysis much faster than the dianion). It was observed that rapid hydrolysis occurred if tetrabutylammonium hydroxide was left out of the reaction mixture or was present in a low concentration relative to the reactant. When [$^{-}$OH] was 50 mM or higher, no hydrolysis was observed after time periods sufficient for the aqueous reaction at the same temperature to proceed to 50% or more of completion.

Kinetic Isotope Effects. The kinetic isotope effects measured for the hydrolysis of pNPP in 90% DMSO at 85 °C are shown in Scheme 1. For comparison, the previously reported isotope effects for the aqueous hydrolysis of the dianion at 95 °C are also shown. Because temperature affects the magnitude of isotope effects, it is desirable to compare data taken at similar temperatures. At 85 °C, the half-life of the reaction is less than 1 min; it was not feasible to conduct the isotope effect reactions at a higher temperature or higher DMSO fraction because of the need to stop the reaction after partial hydrolysis. The temperature-induced deviation expected from a 10° temperature difference is very small.

Computational Studies. Table 1 shows the results of the computational studies of *p*-nitrophenyl phosphate, phenyl phosphate, and methyl phosphate. The results indicate a dependence of the scissile P−O ester bond length on both the leaving group and the solvation of the phosphoryl group. This bond length becomes shorter (and thus the substrate more stable) with increasing solvation or with increasing basicity of the leaving group. The effect of solvation is most prominent in the case of *p*-nitrophenyl phosphate.

Calculations using the ONIOM method were performed on a partially rigid fragment of the enzyme (Figure 3) with methyl phosphate manually docked into the active site in the initial state before optimization, as described in the Experimental Section. The results show a rather surprising, measurable destabilization of the substrate in the alkaline phosphatase active site in comparison to that in solution (Table 1, Figure 7), suggesting a potential susceptibility of the reactant to undergo hydrolysis by a dissociative mechanism.

Discussion

Nature of the pNPP Reaction in DMSO. An indication of the transition state structure can be obtained from the kinetic isotope effects. A considerable body of data has been obtained for the uncatalyzed hydrolysis and the enzymatic reactions of pNPP. The $^{18}k_{\text{bridge}}$ isotope effect gives a measure of the degree of bond cleavage, and the ^{15}k isotope effect is sensitive to charge delocalization in the leaving group.[9,18] The $^{18}k_{\text{nonbridge}}$ isotope effect in reactions of phosphate esters is very small and inverse in reactions with a loose transition state in which the phosphoryl group resembles metaphosphate and becomes normal in mechanisms with more nucleophilic participation such as in the hydrolysis reactions of phosphodiesters and triesters.[9,18,19] A comparison of the isotope effects measured in 90% DMSO solution with those previously measured in aqueous solution is shown in Scheme 1.

The possibility that the DMSO induces a change to a more nucleophilic mechanism is unlikely in view of the unaltered magnitude of $^{18}k_{\text{nonbridge}}$. This conclusion is

(16) Kirby, A. J.; Varvoglis, A. G. *J. Am. Chem. Soc.* **1967**, *89*, 415−423.
(17) Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 3209−3216.
(18) Hengge, A. C.; Tobin, A. E.; Cleland, W. W. *J. Am. Chem. Soc.* **1995**, *117*, 5919−5926.
(19) Hengge, A. C. In *Enzymatic Mechanisms*; Frey, P. A., Northrop, D. B., Eds.; IOS Press: Amsterdam, 1999; pp 72−84.

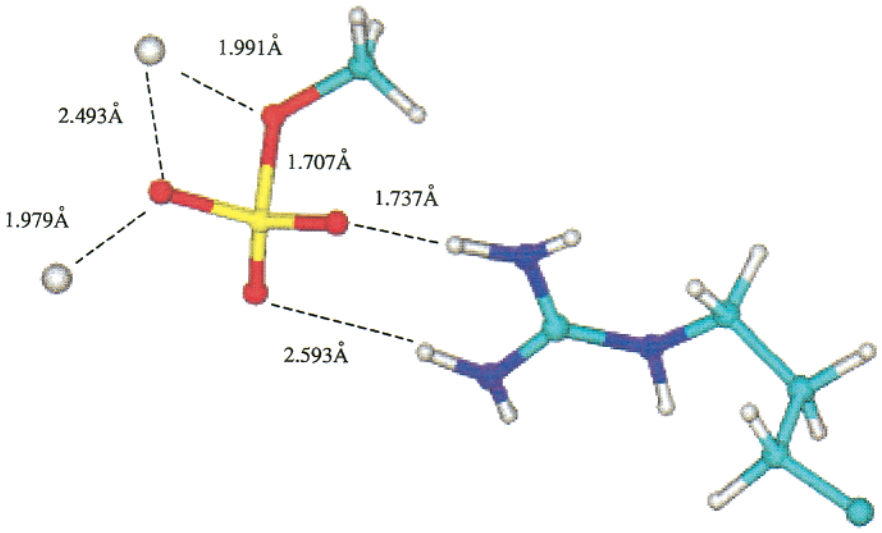


Figure 7. Optimized geometry of methyl phosphate at the alkaline phosphatase active site.

supported by the independence of the hydrolysis rate on the hydroxide concentration. Although the entropy of activation becomes slightly negative in the DMSO/water reaction, the value is still much less negative than values typical of bimolecular reactions such as alkaline ester hydrolysis. Because of the significant effect that solvation can have on entropies of activation, the change in this parameter cannot be confidently ascribed to a mechanistic change and is more likely due to solvation effects.

Both $^{18}k_{bridge}$ and ^{15}k are reduced in magnitude by about one-fourth in the reaction in 90% DMSO. Isotope effects measure the difference between the ground state and transition structure; thus, the reduced magnitudes of these isotope effects are consistent with either an earlier transition state with less P−O bond cleavage or a solvent-induced weakening of the scissile P−O bond in the reactant. In this reaction, the reactant and transition state bear a charge of −2. In the late transition state of the aqueous reaction, this charge is dispersed relatively equally between the phosphoryl group and the leaving group (Figure 1). The high DMSO content results in a less protic solvent compared to water. This should favor a transition state with as much, or more, dispersal of negative charge relative to that of the aqueous reaction.[20] Thus, a significantly earlier transition state, which would have less charge dispersal, would not be expected. However, the Hammond postulate predicts that a destabilization of the reactant, assuming the stability of the product is unaltered, should lead to a somewhat earlier transition state. Thus, it is possible that both effects contribute to the reduced magnitudes of $^{18}k_{bridge}$ and ^{15}k.

Additional information comes from the enthalpy of activation, which in 95% DMSO is substantially reduced from its value in aqueous solution (Table 3). This change is consistent with either destabilization of the reactant, as proposed by Kirby, increased stabilization of the transition state, or a combination of both effects. As stated previously, it is not expected that a dianionic transition state should be better stabilized in the less protic 90% DMSO solvent mixture. The altered KIEs indicate that, in addition to the lowered activation energy, there are changes in the *structure* of the transition state or the ground state relative to the situation in aqueous solution. The simplest explanation of all the data is the one originally proposed by Kirby, a weakening of the P−O ester bond in the ground state at high DMSO concentrations relative to aqueous solution. This would explain both the lowered enthalpy of activation and the reduced magnitudes of the isotope effects in the leaving group. Because of the logarithmic relationship between bond length and bond order, a relatively small increase in P−O bond length can result in significant weakening of the scissile ester bond and reduced KIEs. With application of Pauling's rule,[21] a desolvation-induced increase in P−O bond length of 0.1 Å corresponds to a decrease in bond order from 1.0 to 0.68.

Other Aryl Phosphates. The hydrolysis reactions of *p*-chlorophenyl phosphate, of *m*-bromophenyl phosphate, and of *p*-cyanophenyl phosphate are all substantially faster in 95% DMSO relative to the aqueous reactions. These results with the data for pNPP are shown graphically in the Brønsted plot of Figure 5. If all four points are included in the correlation, the best linear fit has a slope of −1.4; if only the first three points are used, the slope is −1.1. Both values are close to the value of −1.23 reported for the aqueous hydrolysis of a series of aryl phosphate dianions.[16] The similarity in the slopes indicates that the hydrolysis rates of these four aryl phosphates are accelerated to a similar degree.

Phenyl and Methyl Phosphate. For reasons described in the Experimental Section, the effect of DMSO on the reactions of phenyl phosphate and of methyl phosphate were examined in 80% DMSO rather than the 95% used in the reactions of the substituted aryl phosphates. Nonetheless, a rate acceleration of nearly 3 orders of magnitude is expected if the effect of DMSO on these esters is the same as reported for *p*-nitrophenyl phosphate.[6] Hydrolysis occurs if hydroxide is left out, and under such conditions the monoanion is most likely the reactive species (the pK_a of the phosphoryl group will be elevated by the added DMSO). Unexpectedly, the hydrolysis rates of the dianions of phenyl phosphate and of methyl phosphate in 80% DMSO are not accelerated and in fact are slower than the aqueous reactions; the

(20) March, J.; Smith, M. B. In *March's Advanced Organic Chemistry*; Fifth ed.; Wiley: New York, 2001, p 450−451.

(21) Pauling, L.; *The Nature of the Chemical Bond*, third ed.; Cornell University Press: Ithaca, NY, 1960; pp 255−260.

rates were too slow to allow determination of the rate constant. It is intriguing to note from the Brønsted plot in Figure 5, that the point for *p*-chlorophenyl phosphate, which has the most basic leaving group of the set, deviates negatively from the linear behavior of the other three aryl phosphates. The cumulative results demonstrate that the accelerating effect of added DMSO holds only for phosphates having leaving groups less basic than phenol.

It is uncertain why the reaction rate is *slower* for methyl and phenyl phosphate dianions in aqueous DMSO than in water. The extrapolated rate constant for phenyl phosphate hydrolysis in water at 39 °C is close to that expected from a Brønsted plot for aryl phosphates.[16] This rate constant is also very close to that reported for methyl phosphate, which, as previously noted,[12] is orders of magnitude faster that its predicted value. The similarity of the values for the aqueous hydrolysis of these two phosphate esters, despite their different leaving groups, suggests that another mechanism may be operative for the aqueous hydrolysis of phosphate esters having leaving groups with basicity greater than that of phenol. A mechanistic difference might also explain why the accelerating effect from added DMSO disappears for esters with more basic leaving groups. However, the similarity of the entropies of activation for methyl (+3.7 eu),[12] phenyl (+7.3, this work), and *p*-nitrophenyl phosphate (+3.5 eu)[17] hydrolysis are consistent with similar mechanisms. Further work is needed to resolve this question.

Computational Results. The computational examination of the scissile P−O ester bond indicates a trend toward a longer, weaker bond as the leaving group pK_{a-} decreases (Table 1). The same trend is seen in X-ray structures of these phosphate esters.[22−24] (In these structures, the P−O ester bonds are from 0.05 to 0.08 Å shorter than the calculated bond lengths in water. Part of this is likely due to the close contacts made with the ammonium and potassium counterions in these structures.)

The calculations also indicate a trend toward a longer, weaker bond as solvation of the phosphoryl group by hydrogen bonding is diminished. This trend is seen whether one compares aqueous structures with those in DMSO or with the gas-phase structures and is diminished as the leaving group becomes more basic. A difference of 0.1 Å will represent a significant weakening of this bond because of the logarithmic relationship between bond length and bond order.[21] The greatest effect is predicted to occur with the labile substrate *p*-nitrophenyl phosphate, with markedly less of an effect occurring with phenyl and methyl phosphates. This is the same trend seen in the experimental results.

Alkaline Phosphatase. The optimized methyl phosphate molecule in the active site of alkaline phosphatase unexpectedly exhibits a lengthened P−O ester bond (Figure 7, Table 1). Since enzymes are dynamic during catalysis, and evidence has been reported for a conformational change during catalysis,[25] the results must be interpreted with caution. However, they raise the possibility that the hydrogen bonding and dipole−dipole interactions at the active site might be less stabilizing for the phosphate ester dianion than the hydrogen bonding network present in aqueous solution. Such destabilization would favor a loose transition state like that for hydrolysis in solution for phosphate monoesters having good leaving groups.

While the experiments in DMSO/water mixtures suggest that reactant destabilization will be effective only for esters having good leaving groups, it is possible that a phosphatase could take advantage of this mechanism by reducing the pK_a of the leaving group, either by protonation or by complexation to a metal ion. The computational results here only show that such destabilization may be feasible. Further experiments are necessary to determine whether such effects in fact occur or whether they contribute to enzymatic catalysis of phosphoryl transfer.

Conclusions

Both the experimental and computational results indicate that the rate acceleration imparted by the aprotic solvent is limited to phosphate monoesters having leaving groups less basic than phenol. The most likely origin of the rate acceleration is that originally proposed by Kirby, namely, a destabilization of the P−O ester bond induced by loss of solvation of the anionic phosphoryl group. This is supported by the lowered enthalpic barrier, the smaller isotope effects in the leaving group, and the results of computational modeling. The kinetic isotope effects rule out a change to a more nucleophilic mechanism and indicate that the transition state remains loose. The computational results indicate that this desolvation-induced effect is most significant with activated esters and is much smaller with an alkyl phosphate or an phenyl phosphate. However, with phenyl and methyl phosphate dianions, the acceleration from added DMSO is not lost, but the hydrolysis rates are significantly slower than that in aqueous solution. The reasons for this are not clear, but it is pertinent that the majority of what is known about phosphate ester hydrolysis has come from studies of the more labile aryl phosphates.

Acknowledgment. This work was supported by NIH Grant GM47297. We thank Richard Wolfenden for advice on conducting the sealed tube kinetics experiments.

Supporting Information Available: Proton and phosphorus NMR spectra of the sodium salts of *p*-chloro, *p*-cyano, *m*-bromo, and methyl phosphates, and the z-matrices for each of the computational structures listed in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

JO016104P

(22) Jones, P. G.; Sheldrick, G. M.; Kirby, A. J.; Abell, K. W. Y. *Acta Crystallogr., Sect. C* **1984**, *C40*, 550−552.
(23) Garbassi, F.; Giarda, L.; Fagherazzi, G. *Acta. Crystallogr., Sect. B* **1972**, *28*, 1665−1670.
(24) Caughlan, C. N.; Mazhar, U.-H. *Inorg. Chem.* **1967**, *6*, 1998−2002.
(25) Halford, S. E.; Bennett, N. G.; Trentham, D. R.; Gutfreund, H. *Biochem. J.* **1969**, *114*, 243−251.