A Fundamental Assumption about OH⁻ Attack in Phosphate Ester Hydrolysis Is Not Fully Justified

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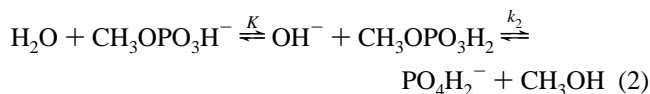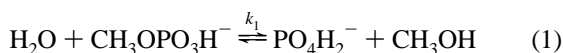*Department of Chemistry, University of Southern California,
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The hydrolysis of phosphate esters is a crucial step in numerous important biochemical processes.[1] This includes the action of G-proteins in signal transduction, energy storage by ATP, and the control of the formation of DNA and RNA. Despite enormous progress in the structural analysis of proteins that control phosphate hydrolysis, we are still not certain what is the exact mechanism of these reactions. This is because the elucidation of mechanistic pathways in enzymes largely depends on our knowledge of reaction mechanisms in solutions at the atomic level. Kinetic measurements provide crucial information about these reactions. However, even this information cannot be interpreted in a unique way.

Information obtained from kinetics studies can be augmented by energy considerations based on quantum mechanical calculations coupled with continuum or semidiscrete models of the solvent. Although the absolute accuracy of calculated reaction rates in solution is still limited, calculations are able to provide reasonable relative values of microscopic rate constants. In the field of phosphate hydrolysis, the importance of solvation effects was pointed out by Hayes et al.,[2] but most computational studies have been limited to gas phase reactions.[3−6] A recent study[7] based on a continuum model of the solvent undermined the hypothesis that strain can account for the increase of the rate of hydrolysis upon going from open chain to cyclic phosphates.

The purpose of this paper is to reevaluate a long-standing mechanistic postulate, namely that it is possible to exclude conclusively the nucleophilic attack of OH⁻ on the neutral phosphate in the hydrolysis of phosphate monoesters, which exist chiefly in anionic forms. The most direct argument for the exclusion of the OH⁻ attack has been based on the comparison of the rates of hydrolysis of methyl phosphate[8] and trimethyl phosphate.[8,9] More specifically, within the family of associative mechanisms, the hydrolysis of methyl phosphate can proceed via the monoanion attacked by a neutral water molecule (eq 1) or via hydrolysis of neutral methyl dihydrogen phosphate by hydroxide ions formed in the pre-equilibrium step characterized by the equilibrium constant[10] $K = 10^{-14}$ (eq 2). Clearly,

$$H_2O + CH_3OPO_3H^- \underset{k_1}{\overset{}{\rightleftharpoons}} PO_4H_2^- + CH_3OH \qquad (1)$$

$$H_2O + CH_3OPO_3H^- \overset{K}{\rightleftharpoons} OH^- + CH_3OPO_3H_2 \overset{k_2}{\rightleftharpoons}$$
$$PO_4H_2^- + CH_3OH \qquad (2)$$

to account for the observed reaction rate constant of 8×10^{-6} s⁻¹ (at 100 °C and pH = 4.2), the second-order rate constant k_2 must be about 10^7 L M⁻¹ s⁻¹,[8] corresponding to an activation free energy, $\Delta G^{\ddagger}$, of about 10 kcal/mol.[11] Unfortunately, k_2 cannot be determined by direct experiments as eqs 1 and 2 have the same kinetic form. (The concentration of neutral phosphate will decrease at high pH, which will exactly counterbalance the corresponding increase in the OH⁻ concentration.)

However, the presumably similar second-order rate constant can be measured for the OH⁻ attack on trimethyl phosphate at high pH, where it amounts to 3×10^{-2} L M⁻¹ s⁻¹ (at 100 °C).[8,9] Since this rate constant is 7 orders of magnitude smaller than the value needed to account for the mechanism in eq 2, it seemed to be justified to reject the involvement of OH⁻ in the phosphate monoester hydrolysis reaction.[8] This conclusion and a closely related conclusion about dimethyl phosphate[12] are in apparent conflict with the recently suggested mechanism for the action of exonuclease domain of Klenow fragment of DNA polymerase I[13] and also with our explanation of observed Brønsted linear free energy relationship in GTP hydrolysis in terms of a mechanism involving the OH⁻ attack at phosphorus.[14−16]

Since it is hard to reach unique conclusions about feasibility of eq 2 by direct experiments, we address this fundamental problem by calculating free energy surfaces for the OH⁻ nucleophilic attack at neutral monomethyl and trimethyl phosphate in aqueous solution. We evaluate solvation energies by using a recently developed ab initio version[17] of our previous hybrid quantum mechanical/Langevin Dipoles (LD) model[18,19] (Figure 1) and also by the PCM model of Miertus and Tomasi[20] implemented in the Gaussian 94 program[21] (Figure 2). The gas phase potential energy surfaces were generated by ab initio MP2/6-31+G** single-point calculations at the geometries optimized at the Hartree−Fock (HF)/6-31G* level. The parameters of LD model were adjusted to reproduce observed solvation energies and absolute pK_a values, which are directly related to the enegetics of hydrolysis. In PCM calculations, generic Pauling's atomic van der Waals radii multiplied by a standard scale factor (1.2) were used. Therefore, there are no adjustable parameters for the transition states in the LD+MP2 model and no adjustable parameters at all in the PCM+MP2 model.

The calculated free energy profiles of the OH⁻ attack at methyl dihydrogen phosphate and trimethyl phosphate in solution are presented in Figure 1 and 2 as functions of the distance between the oxygen of the attacking hydroxyl anion and phosphorus. The free energy of infinitely separated species was taken as a reference point, and reaction coordinates were terminated upon reaching the structure of a pentacoordinated intermediate. These free energy profiles were determined by the LD+MP2 and PCM+MP2 calculations along the reaction

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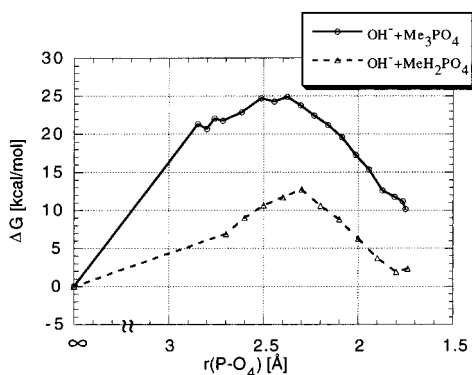(6) Yliniemela, A.; Uchimaru, T.; Tanabe, K.; Taira, K. *J. Am. Chem. Soc.* **1993**, *115*, 3032.
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(11) Derived using the Eyring formula with transmission coefficient equal to 1 and assuming that $\Delta G^{\ddagger}$ for the elimination of the leaving group is equal or smaller than $\Delta G^{\ddagger}$ for OH⁻ attack.
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Figure 1. LD+MP2/6-31+G**//HF/6-31G* free energy profile for the addition of hydroxyl anion to trimethyl phosphate (upper curve) and methyl dihydrogen phosphate (lower curve). Small (± 1 kcal/mol) bumps result from the grid-related uncertainty of the solvation free energies. The total MP2/6-31+G**//HF/6-31G* energies (au) and LD solvation free energies (kcal/mol) are the following: Me_3PO_4, -760.400623, -7.6; OH^-, -75.602058, -109; $MePO_4H_2$, -682.068164, -11.0; TS ($Me_3PO_5H^-$), -836.032858, -73; TS ($MePO_5H_3^-$), -757.729622, -71.
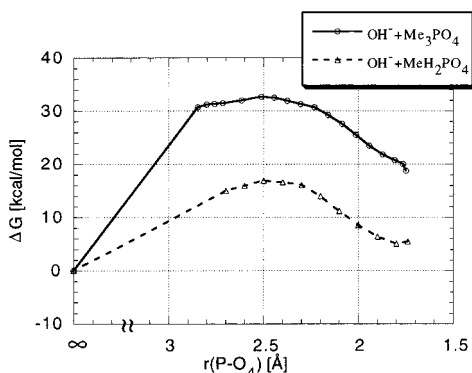
Figure 2. PCM+MP2/6-31+G**//HF/6-31G* free energy profile for the addition of hydroxyl anion to trimethyl phosphate (upper curve) and methyl dihydrogen phosphate (lower curve). This method involves the systematic overestimation of the activation barriers, which originates mainly from overestimated hydration free energy of OH^- ion (-121 kcal/mol calculated versus -108 kcal/mol experimental).
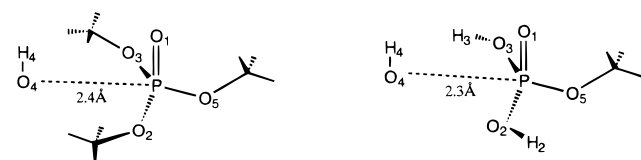
Figure 3. Structure and atom numbering of the transition states (TS) for the addition of hydroxyl anion to trimethyl phosphate (left) and methyl dihydrogen phosphate (right).

coordinates obtained as described below. For trimethyl phosphate, the reaction coordinate was approximated, as the gas phase intrinsic reaction coordinate (IRC)[22] started from the geometry of the transition state. In the monoester case, the productive trajectory of the OH^- attack in solution differs from the fully relaxed gas phase IRC. Therefore, the reaction coordinate for this reaction was obtained as a sequence of partial gas phase geometry optimizations in which the O_3-H_3 distance and $H_2-O_2-P-O_1$ torsional angle (Figure 3) were constrained at 0.95 Å and 89°, respectively, and the $P-OH^-$ distance was increased in steps of 0.1 Å from the value which it attains in the pentacovalent intermediate.

The activation free energy ($\Delta G^\ddagger$) for the OH^- attack at monomethyl phosphate calculated by using the LD solvation

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model (Figure 1) agrees well with the value of 10 kcal/mol required to account for k_2 of 10^7 L s^{-1} mol^{-1}, which was considered by Bunton et al.[8] and others to be unreasonably fast.[23] Also, for triester hydrolysis by OH^- nucleophile, the predicted rate constant[11] of $\sim 10^{-2}$ L s^{-1} mol^{-1} at 100 °C agrees well with the observed value of 3×10^{-2} L s^{-1} mol^{-1}. Thus, for both systems, $\Delta G^\ddagger$ values calculated by using LD model fall in the proper range. More importantly, the calculated $\Delta G^\ddagger$ for OH^- attack at methyl phosphate is significantly lower than for trimethyl phosphate, regardless of the method used for evaluation of solvation contributions. These results indicate that the cleavage of the $P-OCH_3$ bond of monomethyl phosphate by a water molecule may involve an OH^- nucleophilic attack step.

It is not simple to determine the exact distribution of the contributions to this difference in activation free energies. In general, the charge distribution of the P–O atom pair in the $P-O-H$ and $P-O-CH_3$ fragments is quite different and it changes in a different way upon migration of the negative charge of the nucleophile to the phosphate. A more quantitative analysis shows that the formation of the $P-O_4$ bond is assisted at the transition state (TS) for hydrolysis of methyl phosphate by a hydrogen bond involving hydrogen H_3. The gas phase energy contribution of this hydrogen bond is -17 kcal/mol. This accounts, perhaps coincidentally, for the total gas phase difference in activation barriers, $\Delta\Delta G^\ddagger_{gas}$. In solution, this -17 kcal/mol is compensated by 5 kcal/mol due to the difference in solvation contributions to the barrier (44 and 49 kcal/mol for trimethyl and monomethyl, respectively) giving -12 kcal/mol overall difference (Figure 1). When the hydrogen bond is broken in solution, we lose the -17 kcal/mol difference in $\Delta\Delta G^\ddagger_{gas}$, but the solvation contribution reverses (44 and 37 kcal/mol for trimethyl and monomethyl, respectively). This leads to $\Delta\Delta G^\ddagger \approx -7$ kcal/mol from solvation effects. The remaining part (~ -5 kcal/mol) of the total $\Delta\Delta G^\ddagger$ is attributable to hydrogen bonding and possibly other effects. A more complete discussion requires the use of thermodynamic cycles and would exceed the space available for this paper. In addition, we must emphasize that although a qualitative rationalization of the difference in the calculated $\Delta G^\ddagger$ might be insightful, what really counts is that these $\Delta G^\ddagger$ were obtained by realistic calculations. This is different, of course, from the situation before the availability of such calculations when the only way to progress was to use qualitative reasoning for what would have been $\Delta G^\ddagger$ if quantitative calculations were possible. What is essential, of course, is to check whether available experiments are reproduced by the given study, and in the present case, the answer is positive.

In conclusion, the results of the present calculations question one of the most widely accepted assumptions in the field of phosphate hydrolysis. It is found that the rate of OH^- attack on the neutral phosphate monoester is very fast. Previous studies largely underestimated this rate because of the reasonable but apparently unjustified assumption that the rate constant of this reaction is similar to the rate constant of trimethyl phosphate hydrolysis at high pH. This finding suggests that the hydrolysis of phosphate monoesters in aqueous solution is not inconsistent with a mechanism that involves proton transfer to the phosphate oxygen followed by nucleophilic attack on the phosphorus. This is, in fact, the mechanism proposed for the action of G-proteins.[14–16]

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(23) Note that the agreement with experiment further improves when the temperature dependence of the equilibrium constant K (eq 2) is taken into account.