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Theoretical Modeling on the Reaction Mechanism of *p*-Nitrophenylmethylphosphate Alkaline Hydrolysis and its Kinetic Isotope Effects

Violeta López-Canut,[†] Javier Ruiz-Pernía,[‡]
 Iñaki Tuñón,^{*,†} Silvia Ferrer,[§] and Vicent Moliner^{*,§}

Departamento de Química Física, Universidad de Valencia, 46100 Burjassot, Spain, Department of Chemistry, University of Bath, Bath BA2 7AY, United Kingdom, and Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castellón, Spain

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Abstract: We have studied the alkaline hydrolysis of *p*-nitrophenylmethylphosphate (*p*-NPmP) in aqueous solution by means of polarizable continuum models and by hybrid quantum-mechanical/molecular-mechanical (QM/MM) methods. The theoretical predictions of kinetic isotope effects (KIEs) are in very good agreement with the experimental data, confirming a concerted asynchronous molecular mechanism. In addition, comparison of high level DFT theory with semiempirical AM1/d Hamiltonian has allowed checking the reliability of the later to be used in modeling very large molecular models containing phosphorus atoms.

Phosphate esters are fundamental molecules in cellular chemistry.¹ Hydrolysis of the phosphorus–oxygen bond of phosphate esters occurs in biochemical processes including energy storage, biosynthesis or replication of genetic material.² The kinetic stability of the phosphorus–oxygen bond in aqueous solution imposes the use of enzymes to reach these chemical rates compatible with life.³ Thus, to know how enzymes that catalyze these processes (such as kinases, ATPases, and phosphatases) work, it would be interesting to understand the reaction in solution, which is the reference reaction to estimate the performance of these catalysts.

* To whom correspondence should be addressed. E-mail: tunon@uv.es (I.K); moliner@uji.es (V.M.).

[†] Departamento de Química Física, Universidad de Valencia.

[‡] Department of Chemistry, University of Bath.

[§] Departament de Química Física i Analítica, Universitat Jaume I.

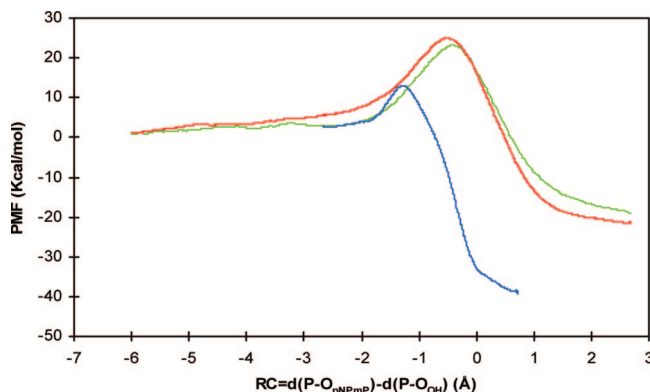


Figure 1. PMF obtained for the hydrolysis of *p*-nitrophenylmethylphosphate (*p*-NPmP) obtained at AM1/MM level at 25 °C (blue line) and at AM1d/MM level at 25 and 95 °C (green and red lines, respectively).

Phosphate esters alkaline hydrolysis may proceed, in principle, with different reaction mechanisms:² (i) a S_N1 dissociative mechanism in which departure of the leaving group originates a metaphosphate intermediate, followed by nucleophilic attack ($D_N + A_N$), (ii) an associative mechanism in which nucleophilic attack precedes the leaving group departure, originating a pentacoordinate intermediate ($A_N + D_N$), or (iii) through a concerted path in which bond breaking and bond forming take place in a single chemical step ($A_N D_N$). Experimental studies based on kinetic isotope effects (KIEs) on phosphate diesters hydrolysis in alkaline solution suggest that this process takes place through a $A_N D_N$ mechanism.^{4,5} Theoretical studies demonstrated that the mechanism becomes more dissociative as the pK_a value of the leaving group is reduced, both for mono- and diesters.^{6,7}

Molecular simulations, compared to experimental measurements of KIEs, provide the adequate framework to elucidate the reaction mechanisms in different environments. In this work, the hydrolyses of *p*-nitrophenylmethylphosphate (*p*-NPmP) (see Scheme 1) in aqueous solution has been studied by means of the polarizable continuum model (PCM)⁸ and by means of hybrid quantum mechanics/molecular mechanics (QM/MM) methods.⁹ In the former, the solvent molecules are described by means of a dielectric continuum and polarizable medium, characterized by a dielectric constant using the Gaussian 03 package of programs.¹⁰ In QM/MM methods, solvent molecules are described explicitly with a box of 55.8 Å side water molecules treated by TIP3P¹¹ potentials as implemented in DYNAMO library.¹²

Keeping in mind that PCM models describe quite accurately homogeneous environment effects, such as aqueous solutions,¹³

Scheme 1

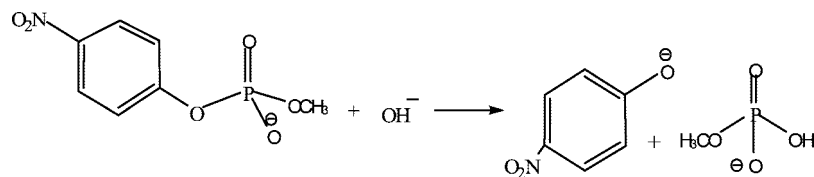


Table 1. Distances (in Å) Defining Breaking and Forming Bonds at the TS Obtained at Two Different Temperatures with the PCM Model and with the Explicit QM/MM (Values Are Reported As Average)^a

	B3LYP(PCM)		AM1/MM		AM1d/MM	
	25 °C	95 °C	25 °C		25 °C	95 °C
$d(\text{P}-\text{O}_{\text{OH}})$	2.516	2.517	2.913 ± 0.058		2.233 ± 0.057	2.403 ± 0.076
$d(\text{P}-\text{O}_{\text{pNpMP}})$	1.851	1.853	1.663 ± 0.036		1.813 ± 0.047	1.858 ± 0.068
$\Delta G_c^{0,\ddagger}$	23.2	25.1	10.3		20.5	21.6
$\Delta G_c^{0,\ddagger}$ (exptl)			25.9 ^b			

^a Free energies of activation are reported in kcal mol⁻¹. ^b Experimental value was obtained at 42 °C from ref 20.

B3LYP density functional theory (DFT) functional, within the 6-31+G* basis set, was used for the PCM optimizations and frequency calculations, while electronic energy was further refined by means of single point calculations using the 6-311++G** basis set. These results will be used as the reference for comparison with QM/MM calculations with low-level QM methods. QM region on these calculations were described with the AM1 Hamiltonian,¹⁴ which is known to not be a sufficiently accurate to model reactions involving phosphorus atoms because it does not incorporate d orbitals, as demonstrated by Marcos et al.¹⁵ in a deep comparison study between different semiempirical methods on pentacoordinated phosphorus TSs involving reactions in gas phase. For this reason, the new AM1/d-PhoT Hamiltonian (hereafter simply named as AM1d)¹⁶ was also used to describe the QM region. Thus, reactants, transition state (TS) and productlike structures have been localized in the PCM approach at 25 °C, as the reference temperature, and 95 °C, the temperature at which the experimental KIEs for this reaction had been measured.⁴ These calculations have been done at the B3LYP(PCM) level by changing the dielectric constant of the solvent from 78.5 to 56.1.¹⁷ Once the TS was localized in the continuum medium, this was used as initial structure to equilibrate the solvent in the QM/MM simulations (both using the AM1 and AM1d methods). The system was relaxed by means of 250 ps of Langevin Dynamics using the NVT ensemble at the two temperatures. A total of 5816 water molecules were present in the 55.8 Å side cubic box at 298 K, while at 368 K only 5576 water molecules were included to have the correct density. The time step employed in all the simulations was 1 fs.

Starting from these structures, the free energy profile of the reaction was then obtained in terms of the potential of mean force (PMF) with the antisymmetric combination of distances defining forming and breaking bond, $\text{RC} = d(\text{P}-\text{O}_{\text{pNpMP}}) - d(\text{P}-\text{O}_{\text{OH}})$, as the distinguished reaction coordinate (negative values correspond to reactants and positive values to products). The different values of the reaction coordinate sampled during the simulations were pieced together by means of the weighted histogram analysis method (WHAM)¹⁸ to construct the full distribution function from which the PMFs were obtained. The value of the force constant used for the harmonic umbrella sampling was 2500 kJ mol⁻¹ Å⁻². Each window consisted of

Table 2. B3LYP(PCM) and AM1d/MM Primary ¹⁸O KIE, Secondary ¹⁸O KIE, and Secondary ¹⁵N KIE for the Hydrolysis of *p*-NpMP in Solution at 95 °C

	B3LYP (PCM)	AM1d/MM	experimental
$(k^{18\text{O}}/k^{16\text{O}})_{\text{p-NpMP}}$	1.0047	1.0044 ± 0.0033	1.0059 ± 0.0005^a
$(k^{18\text{O}}/k^{16\text{O}})_{\text{OH}}$	1.0238	1.0125 ± 0.0054	1.0227 ± 0.0100^b
$(k^{18\text{O}}/k^{16\text{O}})_{\text{ne,p-NpMP}}$	0.9977	0.9966 ± 0.0032	0.9949 ± 0.0006^a
$(k^{18\text{O}}/k^{16\text{O}})_{\text{p-NpMP}}$	1.0029	1.0003 ± 0.0020	1.0016 ± 0.0002^a

^a Values obtained from ref 4, measured at 95 °C. ^b Value obtained from ref 5. See ref 22.

10 ps of equilibration, followed by 15 ps of production, using two reference temperatures of 25 and 95 °C. Resulting PMFs are depicted in Figure 1, while activation free energies and distances defining breaking and forming bonds at the TS are reported in Table 1. It must be pointed out that the free energy barriers were computed as free energy difference between the TS and the fully solvated separated reactants plus a correction at standard concentration 1M,¹⁹ while averaged bonds lengths were obtained from the 15 ps of MD production.

Geometrical and energetic results obtained at 25 °C employing the B3LYP(PCM) and the AM1d/MM methods are in reasonable agreement (see Table 1). Both computational levels describe a concerted but asynchronous mechanism for the hydroxide attack to the phosphate diester. As a reference, we also computed the free energy change associated to the complete breaking of the P-O_{pNpMP} bond at the B3LYP(PCM) level. The resulting free energy at 25 °C is 30.4 kcal/mol, substantially higher than the free energy barriers obtained for the concerted mechanism. We also tried to explore an A_N + D_N mechanism through a pentacoordinated phosphorus species. However it was not possible to locate such a structure either as a minimum or as TS. We have estimated that the free energy cost associated to reaching this quadratic region is about 34 kcal/mol, also higher than the free energy barrier obtained in our proposed concerted mechanism. Thus the A_ND_N mechanism seems to be more likely for this reaction than the D_N + A_N or the A_N + D_N one. This finding is in agreement with a previous work.⁶ The AM1/MM method describes a too early TS, a larger forming bond and a shorter breaking bond. As a consequence, and in accordance with the Hammond postulate, the free energy barrier

appears dramatically smaller, as observed in Figure 1 and in values reported in Table 1. Comparison with experimental barrier obtained by Zalatan et al. at 42 °C,²⁰ seems to indicate that AM1d/MM calculations render much more reliable data than using the standard AM1 semiempirical method. This result is in accordance with inherent limitations on the later to properly describe phosphorus atoms.

The results obtained at 95 °C computed at B3LYP(PCM) and AM1d/MM levels were also in good agreement, confirming the reliability of the AM1d semiempirical Hamiltonian. Both methods describe also a similar reaction mechanism, although the transition structures are slightly more dissociative than at 25 °C. The increase of the activation free energy with the temperature is caused by a diminution of the dielectric effect, which shields the electrostatic repulsion between the reacting fragments and to the negative sign of the activation entropy characteristic of association processes.

To confirm the nature of the molecular mechanism of the reaction, the effects of isotopic substitutions upon the kinetics (KIE) of the hydrolysis of *p*-NPmP in solution have been computed and compared with available experimental data reported in the literature.^{4,5} In particular, primary ¹⁸O KIE for substitution at oxygen atom of nucleophile and leaving group, (k^{16}_O/k^{18}_O)_{OH} and (k^{16}_O/k^{18}_O)_{*p*-NPmP}, respectively, secondary ¹⁸O KIE for substitution at the two nonether phosphorus oxygen atoms, (k^{16}_O/k^{18}_O)_{ne,*p*-NPmP}, and secondary ¹⁵N KIE for substitution at nitrogen atom of nitro group of leaving group, (k^{14}_N/k^{15}_N)_{*p*-NPmP}, have been computed. Calculations have been done within the B3LYP(PCM) and AM1d/MM methods at 95 °C with the CAMVIB/CAMISO programs.²¹ The results, reported in Table 2, were obtained with the rigid-rotor/harmonic-oscillator approximation by computing the Hessian of the atoms corresponding to the full solute as depicted in Scheme 1. For the AM1d/MM values, ten different structures of the transition and reactants states were optimized as first-order saddle points and minima on the QM/MM potential energy surface (PES). These were obtained starting from different configurations of the respective MD productions. These QM/MM calculations were carried out with DYNAMO keeping frozen all atoms far from a sphere of 22 Å centered on the solute. Computed KIEs were obtained as an average over these structures.

The analysis of data reported in Table 2 shows that both B3LYP(PCM) and AM1d/MM methods predict the same kind of KIEs: normal primary ¹⁸O KIEs, slightly smaller for the labeling at the leaving group position, and inverse secondary ¹⁸O KIEs, which is in accordance with other measurements of secondary KIEs for S_N2 reactions. Both methods also render small secondary ¹⁵N KIE on the nitro group at para position of the ring. Thus, the AM1d/MM method seems to be a promising approach to study chemical reactions involving phosphorus atoms in complex environments, such as solutions or enzymes, with the evident advantage of being much less computing demanding than high level DFT methods.

Finally, it can be observed that theoretical predictions are in very good agreement with the experimental data, thus giving credit to the proposed molecular mechanism, a concerted but asynchronous associative hydrolysis of the pNPP in solution.

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evaluated using the PCM model as a result of the average of 10 independent evaluations for structures obtained at $RC = -6 \text{ \AA}$.

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$$\ln(\text{KIE}_{95^\circ\text{C}}) = \left(\frac{310K}{368K} \right) \ln(\text{KIE}_{37^\circ\text{C}})$$

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