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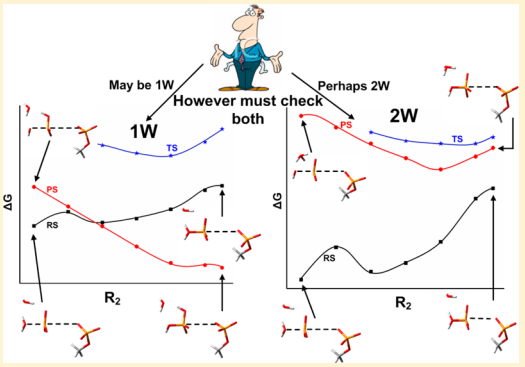
Addressing Open Questions about Phosphate Hydrolysis Pathways by Careful Free Energy Mapping

B. Ram Prasad, Nikolay V. Plotnikov, and Arieh Warshel*

Department of Chemistry, University of Southern California, SGM 418, 3620 McClintock Avenue, Los Angeles, California 90089, United States

Supporting Information

ABSTRACT: The nature and mechanism of phosphate hydrolysis reactions are of great interest in view of the crucial role of these reactions in key biological processes. Although it is becoming clearer that the ultimate way of resolving mechanistic controversies must involve reliable theoretical studies, it is not widely realized that such studies cannot be performed at present by using most existing automated ways and that only careful systematic studies can lead to meaningful conclusions. The present work clarifies the above point by considering the hydrolysis of phosphate monoesters. The clarification starts by defining the actual issues that should be addressed in careful studies and by highlighting the problems with studies that ignore the need for unique mechanistic definitions (e.g., works that confuse associative and dissociative pathways). We then focus on the analysis of the proton transfer (PT) pathways in phosphate hydrolysis and on recent suggestions that PT involves more than one water molecule. Here we point out that most of the studies that found a proton transfer through several water molecules have not involved a sufficient systematic search of the relevant reaction coordinates. This includes both energy minimization approaches as well as a recent metadynamics (MTD) simulation study. To illustrate the crucial need of exploring the potential surfaces reliably, rather than relying on automated approaches, we present here a very careful study of the free energy landscape along a 3D reaction coordinate (RC) exploring both the standard 2D RC, comprised of the attacking and leaving group reaction coordinates, as well as of the proton transfer (PT) coordinate. Our study points out that QM/MM minimization or MTD studies that concluded that the hydrolysis of phosphate monoesters involves a PT through several water molecules, have not explored carefully the single water (1W) path (that involves a direct PT form the attacking water molecule to the phosphate oxygen). Furthermore, we identified the most likely reason for the difficulty in finding the 1W path by QM/MM minimization methods, as well as by the current MTD simulations. We also discuss the problems with current studies that challenge the phosphate as a base mechanism and emphasize that all recent studies found associative/concerted paths (although many have not realized the meaning of their results). Finally, although we clearly do not have the last word about the 1W versus 2W paths we believe that we illustrated that the crucial mechanistic problems with alternative pathways should not be resolved by just running black box search approaches.



I. INTRODUCTION

Phosphate hydrolysis reactions are arguably the most important class of biological reactions,[1−7] and it is thus important to elucidate the mechanism of such reactions in solution and in proteins. In this respect, it is now gradually realized that quantum mechanical and combined quantum mechanical/molecular mechanics (QM/MM) studies are most probably the only way of resolving the fundamental mechanistic controversies associated with phosphate hydrolysis. However, this does not mean that just running uncritical black-box QM/MM-based calculations can be used to resolve any deep mechanistic issues. In fact, it seems that some in the theoretical community have not yet gained the perspective and experience of the experimental community, where it is a common practice to look carefully at all possible alternative options. Instead, it is sometimes assumed that the results obtained by a specific computational method must represent the reality. The problem with uncritical theoretical studies of phosphate hydrolysis by many irrelevant gas phase studies[8−10] and subsequently by some energy minimization studies[11] are rather obvious (see the discussion in ref 12). Furthermore, even recent studies that involve some configurational sampling[13−15] are not sufficient for obtaining a reliable mechanistic picture. This is largely due to the fact that these studies have mainly focused on exploring limited mechanistic pathways rather than exploring all possible options. This can be very problematic especially when there are several earlier proposals in the literature (e.g., refs 12, 16, and 17). In the case of phosphate monoester hydrolysis, the debate involves two issues: first, it is not clear whether the hydrolysis follows an associative or a dissociative path (unfortunately,

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most of the studies failed to identify the clear distinction between these two), and second, it is not clear whether the proton transfer (PT) from the attacking nucleophilic water molecule to the terminal phosphate oxygen occur in a direct manner or with the assistance of several water molecules. Apparently it is clearly not sufficient (or useful) to explore a PT between several water molecules while not examining carefully a transfer from a single water molecule. Thus, it is important to try to clarify what are the problems and to present a clear benchmark that can be examined by those who are truly interested in the nature of phosphate hydrolysis reactions.

The basic issues in the mechanism of the hydrolysis of phosphate monoesters and in related systems can be formulated clearly and uniquely by considering the structural models presented in Figure 1, where R_1 represents the nuclear

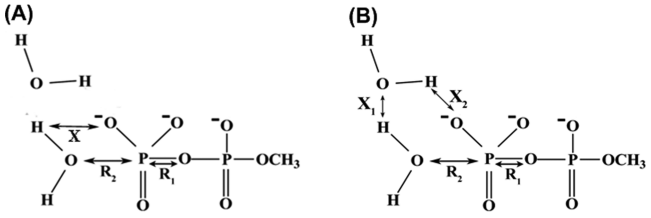

Figure 1. Structural models used in this study to demonstrate the hydrolysis of monomethyl pyrophosphate trianion in solution. R_1, R_2, and X define the three reaction coordinates, which explicitly will be varied during the course of the reaction. Panel A represents the structural model where the proton has been directly transferred from the attacking nucleophilic water molecule to the substrate phosphate oxygen atom (direct PT (1W) mechanism). Panel B represents the structural model where the proton transfer occurs through the assistance of an additional water molecule (water assisted PT (2W) mechanism).

reaction coordinate between the terminal P and the leaving O atoms, R_2 describes the nuclear reaction coordinate between the terminal P and the attacking nucleophilic O atom, and the PT reaction coordinate is designated by X. As described above, the PT can occur along the associative or dissociative paths in either a direct manner (1W) or with the assistance of the second water molecule (2W) (this case is also taken as a representation of PT that involves even more water molecules). Both of these cases are depicted in Figure 1, panels A and B, respectively. Traditionally, the transition states of phosphate hydrolysis have been classified as associative or dissociative, depending on the relative distances of R_1 and R_2. This definition is related to the More O'Ferrall-Jencks (MOFJ) type surfaces[17] which are illustrated in Figures 2 and 3 for 1W and 2W cases, respectively. The representative structures corresponding to the characteristic points all along the various reaction pathways are also depicted explicitly in Figures 2 and 3 for the respective cases. The reaction coordinates R_1 and R_2 appear to provide a more unique picture than that obtained by using bond orders or Leffler indices such as those used by Williams et al.[18] The requirement for a unique formulation cannot be overstated as softer definitions lead to major confusions with clear terms like associative and dissociative being replaced by "loose" and "tight" terms[19−23] (see the discussion in ref 12). Here it is crucial to insist on keeping the original definition according to the shape of the surface. With this in mind, we can move to resolve the main current valid controversies (after clarifying common confusions).

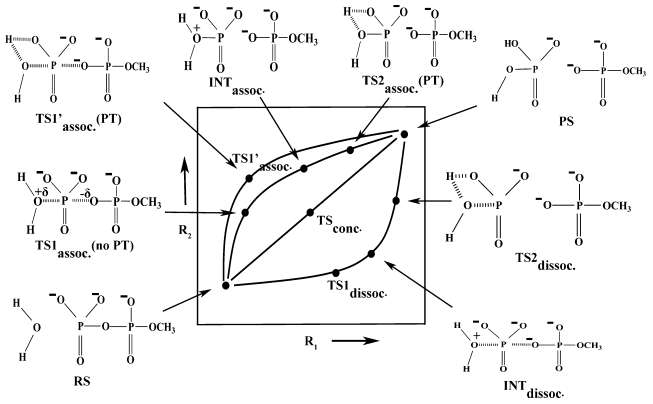

Figure 2. Schematic description of the potential energy surface for the hydrolysis of monomethyl pyrophosphate trianion utilizing the direct PT mechanism. The figure provides a clear definition of the associative, dissociative, and concerted pathways. Representative geometries corresponding to the key characteristic points along the key reaction pathways also depicted. Note that for simplicity we do not show here the additional water molecule that is involved explicitly in the 2W process .

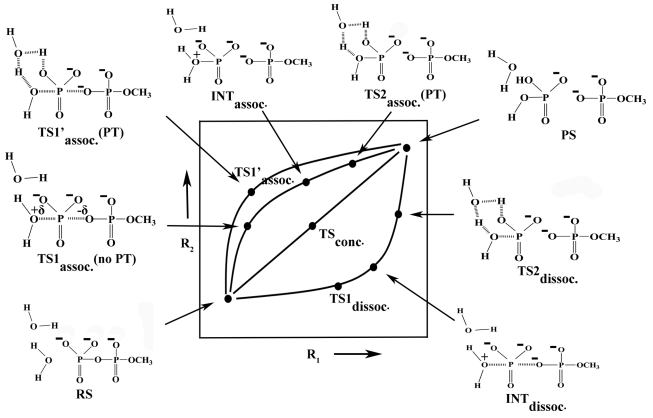

Figure 3. Schematic description of the potential energy surface for the hydrolysis of monomethyl pyrophosphate trianion utilizing the water assisted PT mechanism. The pathways corresponding to the associative, dissociative, and concerted mechanisms are clearly defined and the representative geometries corresponding to the key characteristic points along the key reaction pathways are also depicted.

Arguably, the most extensive and systematic computational studies of the relevant surfaces found[12,16,17] a similar barrier for the associative and concerted paths and with a proton transfer from the attacking water molecule to the phosphate oxygen (see Figure 2). Now, ignoring the irrelevant and problematic works discussed in ref 12, we will focus on a few recent studies that actually have presented some alternative views. This is done with the help of Figure 4 that temporarily "avoids" the difficulties of talking on a specific potential surface by focusing on the paths between different configurations.

We will start our discussion with the mechanistic pathway explored by Nemukhin and co-workers,[11,24] who explored the hydrolysis of triphosphate and GTP in an aqueous solution and in the Ras/GAP respectively. These works involved energy minimization on a QM/MM potential with a relatively low level of theory for the QM part. The minimization in condensed phases raises certain concerns regarding the energy convergence due to the presence of multiple local minima encountered in minimization in addition to the accuracy of

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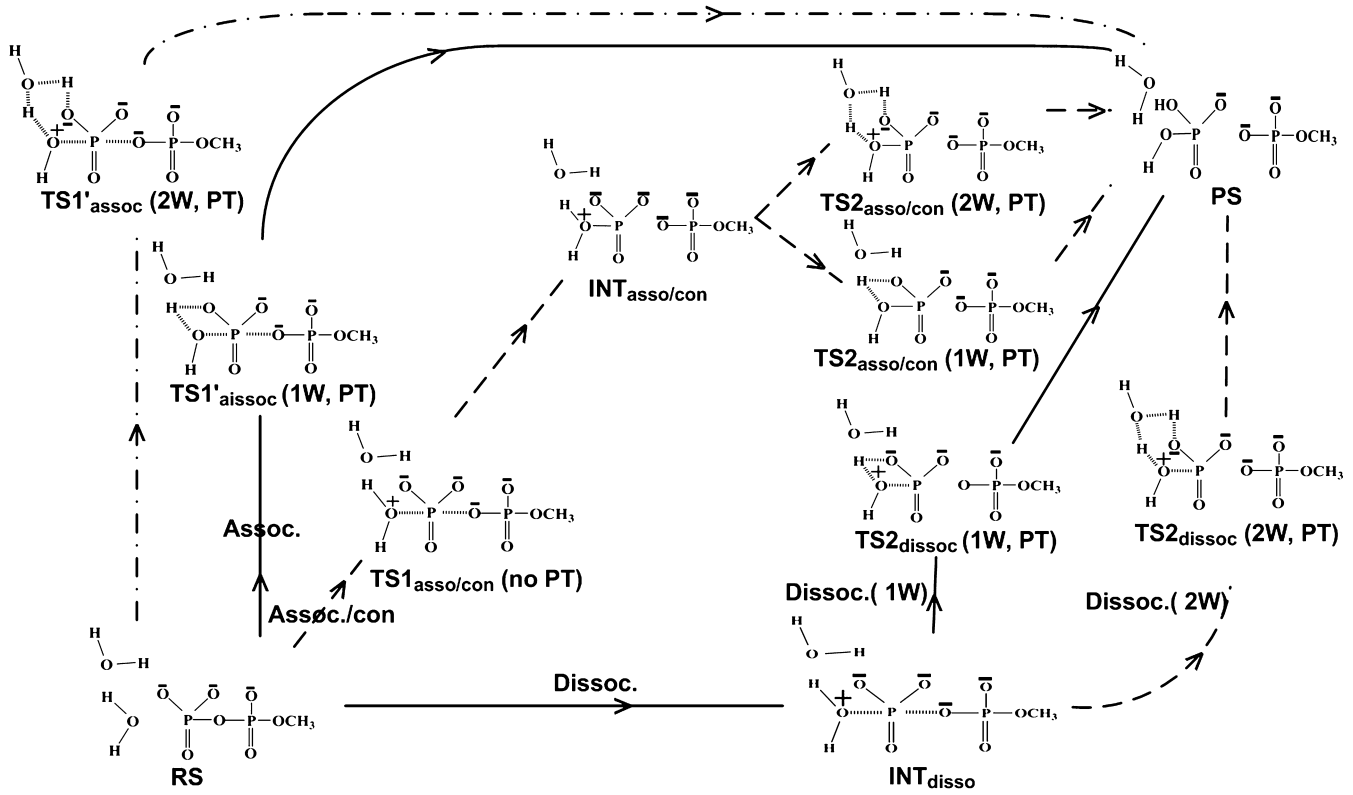

Figure 4. Schematic description of all of the probable mechanistic pathways through which the hydrolysis of monomethyl pyrophosphate trianion could take place. Starting from RS, the geometries of all of the key points along the associative and dissociative pathways following the direct (1W) and water assisted (2W) PT mechanisms are also presented. The central path through TS1assoc (no PT) can also be used to describe a more concerted path.

the prediction of the barrier heights, as well as the transition state geometries, by the low-level QM. These issues are even more serious when it comes to QM/MM studies of proteins (see ref 25 for detailed discussion). Nevertheless, the findings of ref 11 with regard to the solution reaction are of a significant interest. That is, these workers were the first to point out that the attacking water arrives head on (as is the case in Figure 1), which leads to the formation of the $H_2O^+-PO_3^{2-}$ intermediate[11] (Figure 5 in our study). Here, it is interesting to note that, during the formation of this intermediate from the RS, the geometric coordinate, R_1, is varied from approximately 1.68 to 2.3 Å, whereas R_2 is varied from 3.2 to 2.3 Å. This clearly suggests that the reaction follows an associative path (or associative/concerted path) at least up to the rate determining TS. Unfortunately, it was described as a dissociative path (see similar problems in refs 13 and 26 those will be discussed further below). Furthermore, the calculated PT barrier is not the rate limiting barrier since it is not the global maximum along the reaction path. At any rate, the main presumption in this study has been that the PT involves several water molecules in TS2, i.e., during the PT step (Figure 3 of ref 11 and the designated path in our Figure 5), rather than a single water molecule.

A more recent study on the hydrolysis of phosphate monoesters has been reported by Marx and co-workers,[13] who used the metadynamics (MTD) approach (with ab initio (BLYP-based) MD) used to construct the relevant free energy surface. Although the computational cost of the BLYP functional with the plane waves allowed them to simulate a large QM system with 113 water molecules, without proper spherical boundary conditions, it may not be enough to capture

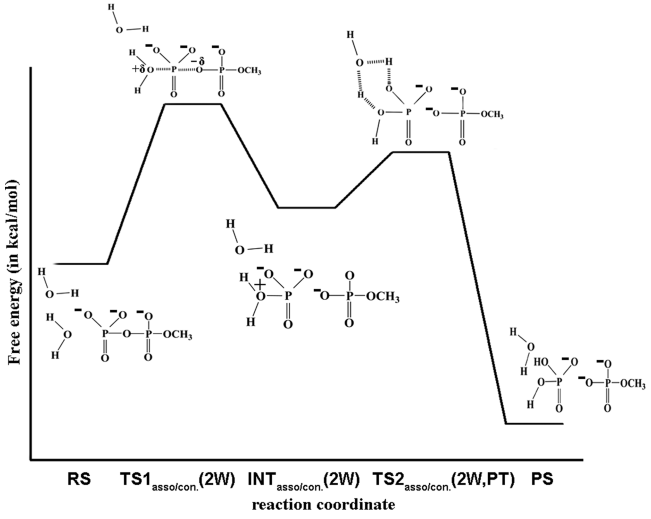

Figure 5. Schematic diagram of the free energy surface and the representative geometries for the hydrolysis of monomethyl pyrophosphate trianion in solution following the catalytic mechanism explored in ref 11. This mechanism corresponds to the asso/con (2W) pathway depicted in Figure 4.

the long-range electrostatics and to properly describe the solvation. Similar problems are likely to exist even with periodic boundary conditions (PBC), except in simple cases with a central isolated charge where clear corrections are available (see the discussion in ref 27). Furthermore, the performance of the BLYP functional in evaluating activation free energy barriers for chemical processes is relatively poor.[28] Here again the actual

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reaction path was clearly found to be the concerted (see Figure 4 in ref 13), but unfortunately the authors who call it "dissociative" ignored this fact. More significant was the finding that the heights of TS2 and TS1 are very similar (about 29 kcal/mol) and thus the suggested mode of the PT might now be rate limiting (in contrast to the case of ref 11). However, it is not clear that the coordination numbers used as the MTD collective variables (CV) in the study of ref 13 actually explored a direct 1W PT pathway (since the authors leave validation of the sophisticated computational scheme to the reader), and this issue will be explored below. Furthermore, the nature of the PT path is important, since Marx et al.[13] presumed to find a new mechanism (basically that of Grigorenko et al.[11]) which they described as a "water as a base" mechanism. The apparent problem is that if TS2 is actually lower than TS1 (the possibility depicted in Figure 5) the presumption of the new mechanism is not justified since the rate-determining step involves a nucleophile attack of a water molecule and not the proton transfer step, where the proton ends up eventually on the phosphate oxygen (thus the phosphate is the actual base (see section V)). At any rate, if the energy of TS2 is higher than that of TS1, then it is still essential to explore the relative energy of the 1W and 2W paths until the actual rate limiting step is reliably determined. To clarify the above problems and to establish the basis for a well-defined analysis, we provide in Figures 2, 3, and 4 a clear (though not trivial) description of the mechanistic alternatives.

We would also like to clarify that a path through which we move to a given TS defines the mechanism much more than the position of the TS or the intermediate. For example, the intermediate (INT) in Figure 3 could have been obtained with a dissociative path or associative path. In the work of refs 11 and 13, it was obtained through an associative path, and this should be used in describing the reaction as an associative reaction. With the above problems in mind, we will provide in this work a very systematic study of the 1W and 2W paths and will do so in a way that can be easily verified by other workers in the field.

II. COMPUTATIONAL METHODS

This work explores the free energy surface of phosphate monoester hydrolysis by a reasonable and well tested approach that can be reproduced by others. Our task is to map the reaction free energy surface in solution in a way that does not miss key points on this surface (missing such points is common to automatic energy minimization approaches). The challenge is to explore the surface while constraining the minimum number of essential coordinates and yet to be able to go against the gradient along the least energy path . The most obvious search involves the coordinates R_1 and R_2 (as described in Figure 1) that fully define the associative and dissociative paths. Unfortunately, it is also essential to explore the third coordinate, namely the proton transfer coordinate, X in case $\partial E/\partial X \gg 0$. That is, if we had a perfect and complete search method we could have assumed that we can locate the least energy path with respect to all essential coordinates in the complete configurational space. However, it is clear that standard energy minimization and probably nonsystematic sampling approaches, (including the MTD approach) cannot do so in the 1D ($R_1 - R_2$) or 2D space of R_1 and R_2 (at least without an adequate description and a special focus on the PT coordinate). In fact, as recognized by Guthrie,[29] we should discuss the surface of phosphate hydrolysis by diagrams of the type presented in Figure 6. Furthermore, considering only R_1 and R_2 can lead to the equivalent of missing nonequilibrium

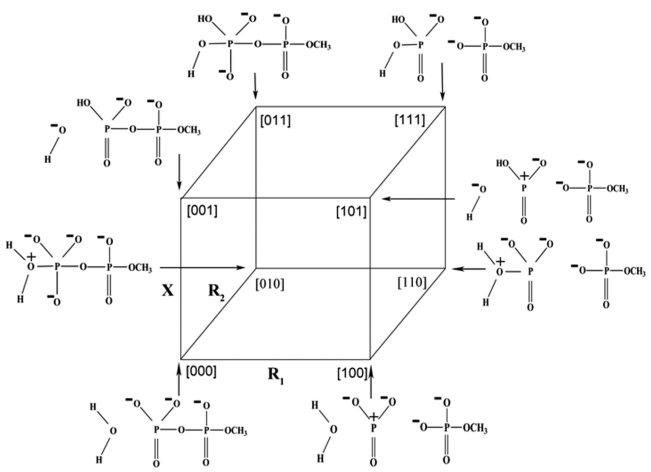

Figure 6. Schematic three-dimensional representation of the hydrolysis of monomethyl pyrophosphate trianion along the three reaction coordinates R_1, R_2, and X. [000] and [111] represent the RS and PS states, respectively. [100], [010], and [001] are the states representing where the reaction had proceeded only along that respective reaction coordinate. [110], [101], and [011] correspond to the states where the reaction had occurred along the respective two reaction coordinates.

solvation effects[30] and to significantly underestimating the contribution of the PT to the overall barrier as it does not proceed along the least-energy path involving an uphill PT at earlier stages. Thus, it is useful to start by exploring the dependence on R_1 and R_2 for different fixed values of X and also with well-defined constraint transition between several water molecules (see below). With the above perspectives in mind we focused on mapping the solution free energy surface for the hydrolysis of mono methyl pyrophosphate trianion in 3D space using ab initio calculations with implicit solvent models as described below.

All of the ab initio calculations were carried out using the *Gaussian 03* software package.[31] The initial geometry optimizations were performed in the gas phase using the 6-31+G* basis set and Becke's three level hybrid functional which combine the Hartree−Fock exchange and density functional theory (DFT) exchange correlations.[32] The resulting structures were described using the larger 6-311++G** basis set and solvated by the IEF-PCM continuum model.[33] The calculated free energy surfaces were subsequently used to identify the approximate location and energies of key stationary points. In addition, the performance of the IEF-PCM continuum model in estimating the solvation influence on the gas phase optimized geometries was compared with that of the COSMO continuum model at the characteristic points on the PES. The error range associated with the choice of the quantum method and the basis set was also established by evaluating selective points at the MP2/6-311++G** level of theory.

Obviously, it is important to have a proper minimization of the system in a given solvent model. Here we note that the mapping in the space of the three key coordinates reduces significantly the need of a careful mapping, since the key coordinates are included in the systematic search (and are not needed to be minimized) . However, we still have to attempt to combine our mapping with more systematic minimization in

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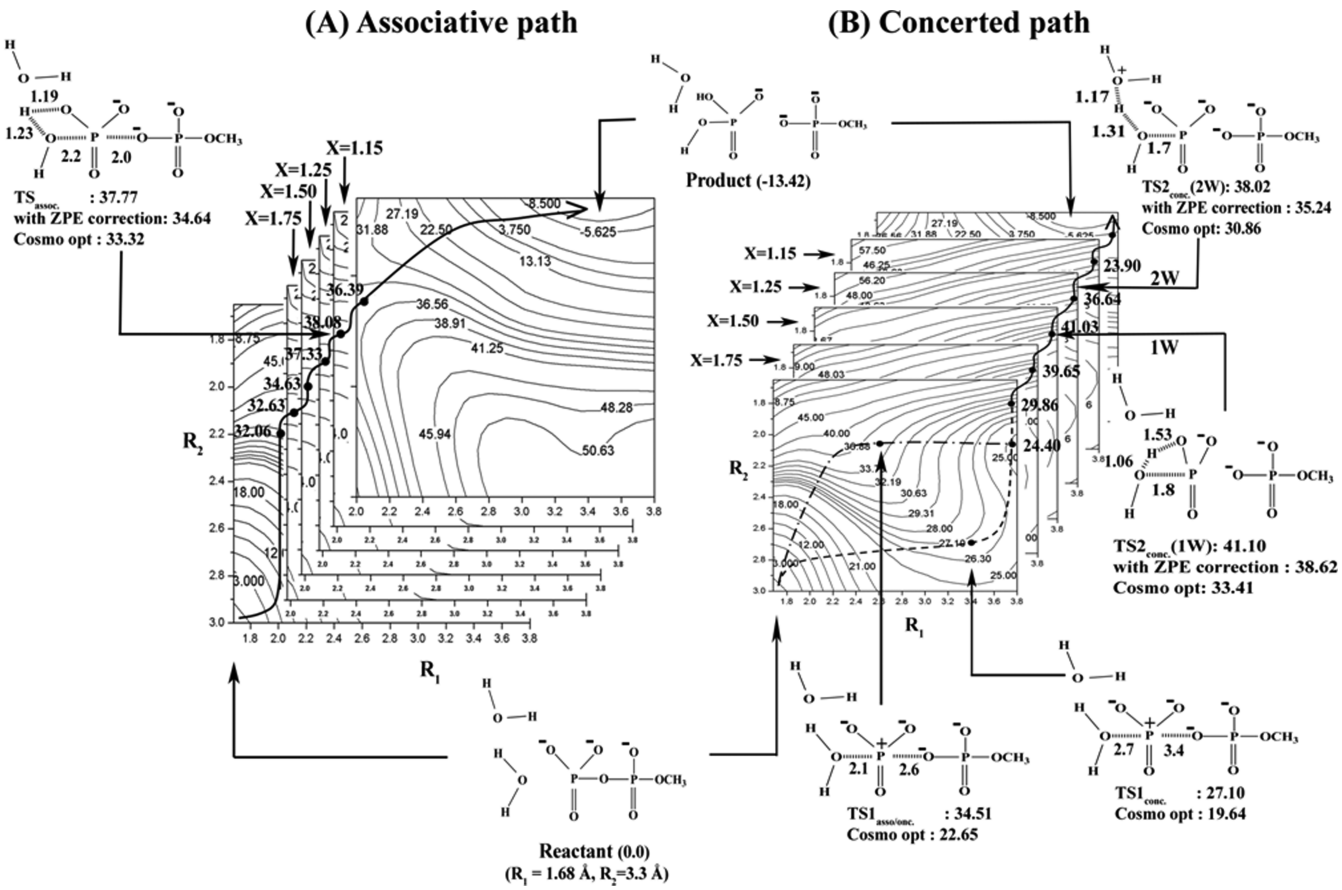

Figure 7. Calculated free energy surfaces along the associative (A) and concerted/late PT (B) pathways for the hydrolysis of monomethyl pyrophosphate trianion in solution utilizing both the 1W and 2W mechanisms. As seen from the figure the rate determining TSs for the 2W and 1W have similar energies. The surfaces are drawn as functions of R_1 and R_2 for several values of proton transfer coordinate X (R_1, R_2, and X are defined in Figure 1A). TS_{assoc} describes the transition state along the associative pathway, and $TS1_{conc}$ and $TS2_{conc}$ describe the transition states along the concerted pathway. The values of the TS energies evaluated by different approaches are given near the corresponding TSs figures. Note that the figure also provides the COSMO optimized TSs which are not always where the initial search indicate them to be. However, the search provides a powerful guide for the relevant regions. Furthermore, note that the critical TSs ($TS2_{conc}$(1W) and $TS2_{conc}$(2W)) were carefully explored. However, because the transition states $TS1_{asso/conc.}$ and $TS1_{conc}$ are neither associated with the rate limiting steps of the reaction nor involved with the critical proton transfer step, they were only identified in a tentative way based on the energetics along the minimum free energy pathway. The figure represents the 1W map, while the 2W TS is placed at the corresponding position of the 2W map. It is possible that more concerted 2W path will have lower barrier but this issue should be explored by the PD approach.

the given solvent model. To achieve this we adopted some aspects of the strategy used in our early work[16] (where we locate a gas phase transition state and then search in the direction of the IRC in solution) and combined it with the 3D mapping technique previously used in other studies by our group.[34] That is, we took the following three step procedure (i) we performed gas phase energy minimization at each point on the 3D map and then added the solvation energies of the corresponding geometries by using an implicit IEFPCM model.[33] (ii) We took points that look like good candidates for being in TSs regions (or key intermediates) and performed gas phase TS optimization and then reevaluate the energy of solvating the resulting structures in the implicit solvent model. (iii) We selected important regions on the surface (namely intermediates and TSs regions) and performed "solvation focusing" by taking a limited grid of the available (R_1, R_2, and X) space and then performing COSMO[35,36] minimization thus exploring the effect of relaxing the remaining solute coordinates in solution. We must note that the TSs obtained in implicit solvent models are far from being perfect, and we believe that more advanced exploration should involve PD approach.[37,38]

III. RESULTS

Our main test system was taken as monomethyl pyrophosphate trianion in solution, where in order to explore in a reliable way the free energy surface for the hydrolysis of this system we started with the systems shown in Figure 1 with two explicit water molecules and explored the 1W and 2W paths following the approach outlined in section II. Our exploration of the free energy surface started by generating a 2D surface in the space of R_1 and R_2. This was done for a series of fixed distances of the PT coordinate X. As stated above, we allowed all of the coordinates except R_1, R_2, and X to be optimized freely at each point (first in the gas phase and then in a more limited way in solution). The reaction coordinate corresponding to the bond breaking, R_1, is mapped in the range from 1.8 to 3.8 Å in 0.2 Å increments. In addition, the energetics corresponding to R_1 = 1.68 Å has also been computed for the entire range of R_2. Similarly, the reaction coordinate corresponding to the bond formation, R_2, was mapped in the range from 3.0 to 1.65 Å in 0.2 Å increments. However, in the range between R_2 = 2.2 and 1.8 Å, the mapping was carried out in 0.1 Å increments for extra clarity. The mapping along the proton transfer coordinate, X,

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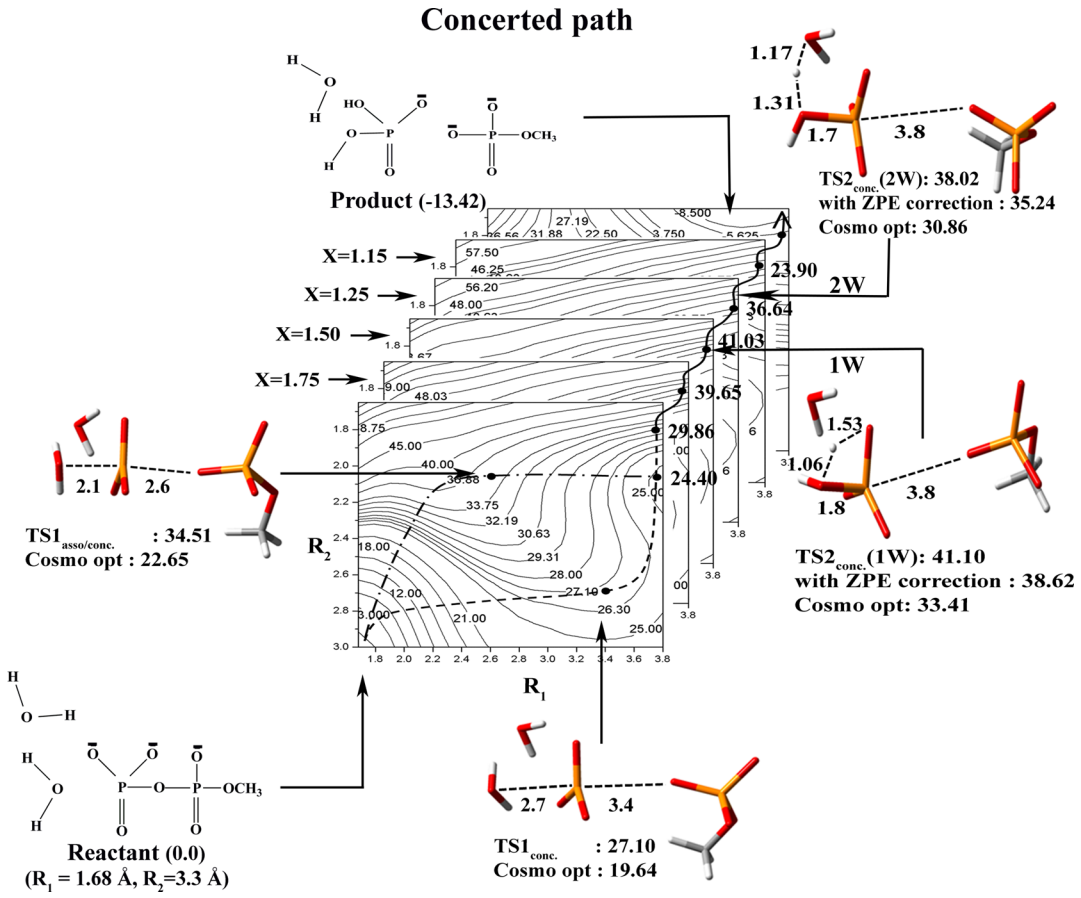

Figure 8. Focusing on the calculated free energy surfaces for the reaction paths that starts with an associative or a dissociative path followed by a late PT to the phosphate oxygen. As seen from the figure, the rate determining TSs for the 2W and 1W paths have similar energies. Note that the activation entropy of the 2W path (which is not included) should be larger than that of the 1W barrier. The surfaces were obtained as functions of R_1 and R_2 for several values of the proton transfer coordinate X, and it focuses on comparing the PT barriers that start from the intermediate where R_2 is around 2 Å and R_1 is partially broken. Note that the figure also provides COSMO optimized TSs which are not always where the initial search indicate them to be. However, the search provides a powerful guide for the relevant regions. The figure represents the 1W map, while the 2W TS is placed at the corresponding position of the 2W map.

was carried out from the reactant state, where the proton is bound to the nucleophilic water molecule, to the product state, where the proton is fully transferred to the leaving phosphate group, through the intermediate states at X = 1.75, 1.50, 1.25, and 1.15 Å. Considering all six 2D free energy surfaces (each of which corresponds to a specific location of the proton, i.e., water bound, protonated phosphate, and intermediates) allowed us to identify the minimum free energy path for the hydrolysis reaction in the hyperspace of R_1, R_2, and X. We note in this respect that, by generating the surfaces in this manner, it has been crucial to change the reaction coordinate very carefully, to avoid unwanted noise on the free energy surface that could obscure the location of the key stationary points. After this initial exploration, we performed a gas phase transition states search in the regions where the actual TSs may exist, and then we performed further focusing by selective COSMO minimization (see section III).

The results of our calculations are summarized in Figures 7−11. As seen from Figures 7 and 8, the 1W and 2W mechanisms have similar activation barriers, and thus it is unjustified to assume that the 2W (or 3W) mechanisms are favored in solution. The reason why the 1W mechanism might have been overlooked in other studies will be clarified below. The structures of the key TSs are provided in Figure 9 and their

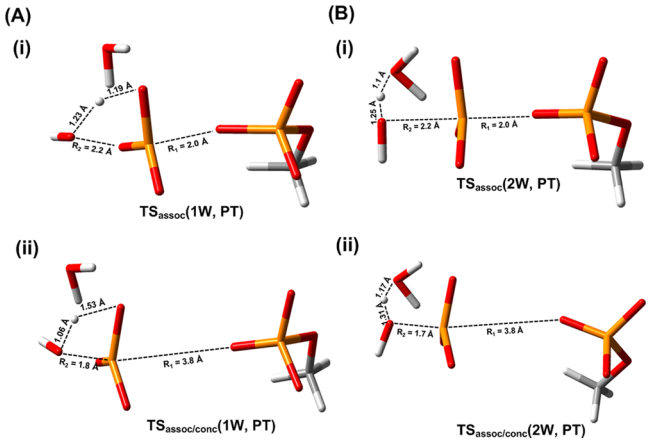

Figure 9. Geometric features of the key transition states corresponding to the 1W and 2W pathways following the associative and associative/concerted mechanisms.

corresponding Cartesian coordinates are presented in the SI. We also provide in the SI an assessment of the effect of the level of the QM and solvation models used. The above study has been done for the reaction in solution without Mg^{2+} ion. This corresponds to the study of Grigorenko et al.,[11] whereas

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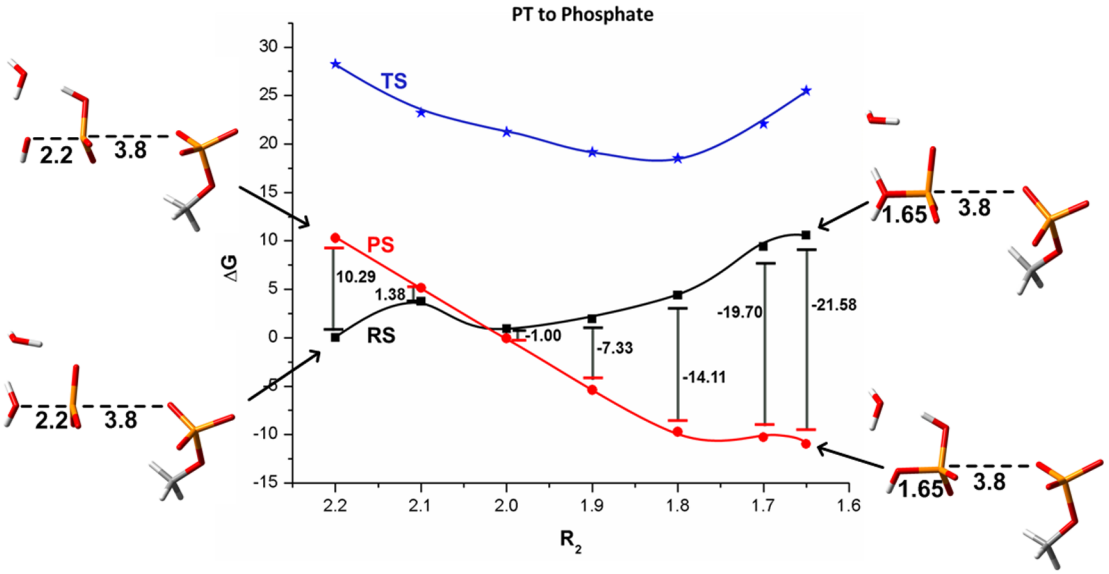

Figure 10. Effect of the change in pK_a of the attacking water on the PT energetics in the 1W mechanism. The figure displays the R_2 dependence of the energy of the "reactant state" (RS), where the proton is bound to the nucleophilic water and the water oxygen is in a short distance of 2.2 Å, the "product state" (PS), where the proton is attached to the acceptor phosphate oxygen, and the corresponding TSs for the PT process. As discussed in the text and shown in Figure 11, the attacking water becomes much more acidic upon formation of the O−P and the PT occur. However, the PT does not become spontaneous because of the extra energy required for bending the O−P−O angle for an optimal PT.

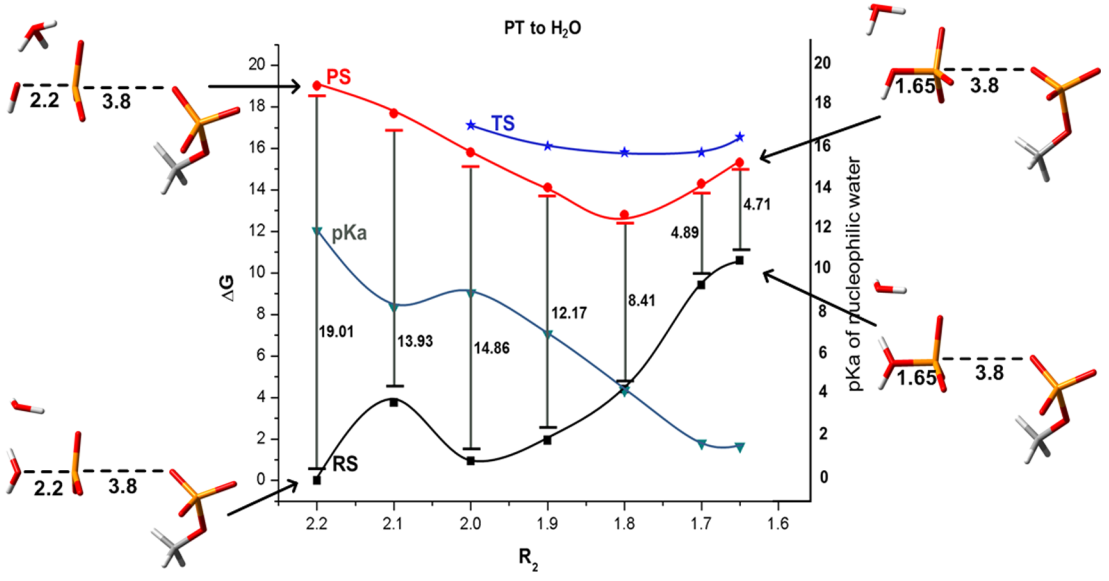

Figure 11. Effect of the change in pK_a of the attacking water on the PT energetics in the 2W mechanism. The figure displays the R_2 dependence of the energies of the "reactant state" (RS), where the proton is attached to nucleophilic water, the "product state " (PS), where the proton is attached to the second water, and the corresponding TSs for the PT process. As seen from the figure, the attacking water becomes much more acidic upon formation of the O−P and the PT becomes basically spontaneous. Note that our constrained mapping does not capture the exact least energy path and overestimates the TS energy .

the study of Marx and co-workers[13] considered Mg^{2+} assisted hydrolysis, a system that was also considered in our previous (Figure 9 of ref 17) study. Our studies of the difference between the reaction with and without Mg^{2+} have indicated that we have a similar trend and we do not expect to see a significant difference between the 1W and 2W cases due to the Mg^{2+} effect. Obviously it would be instructive to perform the free energy calculations with a suitable ab initio QM/MM (QM(ai)/MM) potential using the paradynamics (PD) reference potential-based approach,[37,38] and such a study is in progress in our lab. However, at present it seems to us that the relative energies of the 1W and 2W mechanisms are unlikely to change significantly.

IV. WHY HAS THE 1W PATH BEEN MISSED AND WHAT IS THE REASON FOR SPONTANEOUS 2W MOTION WITH COMPRESSED BONDS?

When suggesting that different studies overlooked key reaction paths, it is crucial to find out the reasons for such problems. Here we explore the reason for overlooking the 1W path by energy minimization (and MTD) studies by evaluating the energetics of the PT at different steps of the nucleophilic attack

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(different values of R_2). The results are presented in Figures 10 and 11, which provide a remarkable new insight. That is, it appears that the compression of the O−P distance drastically changes the pK_a of the attacking water (from about 16 to approximately zero). Obviously at some stage the attacking water becomes very acidic and transfers its proton to either another water or to the phosphate. The problem is that the compression free energy costs must be taken into account. Now adding the effect of the change in pK_a between the donor and acceptor to the compression energy gives the free energy of the "reactant" and "product" in the PT process (see Figures 10 and 11). However, what we actually need is the free energy of reaching the TS for the PT process. Here we have the main difference between the 2W and 1W. That is, in the case of 2W the exothermicity and the TS energy are almost equal, since the acceptor water molecule can be arranged in an optimal distance. This means that change in R_2 during an automated energy minimization or MTD will reach eventually a point with a spontaneous PT. This however not the case for the 1W path. In this case the energy will always go up along the PT coordinate since it is necessary to change the formed P−O and/or bend the O−P−O angle for an optimal PT. The barrier for the PT in the case of 1W is almost certainly the reason why this path has been overlooked by energy minimization and MTD studies. Thus it seems to us that the hydrolysis reaction requires a more complex RC search, which is able to capture reliably uphill PT.

V. DISCUSSION

Phosphate hydrolysis of monomethyl pyrophosphate trianion in solution is a prototype solution reaction for several important biological processes such as the catalytic reactions of GTPase and ATPase. However, there exist several proposals in the literature about the hydrolysis of this class of system and especially about the proton transfer step. We have found in several cases that the proton transfer during the reaction can take place directly from the incoming nucleophilic water molecule to the substrate oxygen atom of the phosphate group. However, there are several other proposals, where the proton transfer occurs through the assistance of additional water molecules rather than a direct transfer mechanism. Now, although any mechanistic proposal based on a proper scientific background deserves to be considered, it is important to try to prove that it is the most probable operational mechanism for the given reaction. Thus one needs to show in an unbiased way that the corresponding rate limiting free energy barrier is the lowest one available. Unfortunately, there is a risky bias in attempting to propose phosphate hydrolysis mechanisms with a path through several water molecules without exploring the alternative pathway. This problem has been compounded in different attempts[11,24,39,40] to explore (and dismiss) the 1W, and phosphate as a base mechanism, while examining an early PT to the phosphate oxygen before the reduction of R_2, rather than exploring the concerted path used in our studies (e.g., ref 41). We would also like to mention that the idea of multiwater PT in phosphate hydrolysis gain major popularity[22,42−44] in recent years, probably due to the popularity of the Grotthuss mechanism, although the role of this mechanism (and in particular the role of water orientation) has been found to be very problematic in many cases of proton transport in proteins (e.g., see ref 45). Here again we like to point out that it is completely valid to suggest such path but it is also essential to explore the free energy of the single water path. Finally, traps associated with insufficient attention to the PT coordinate have also been discussed recently in ref 46.

It might also be useful to comment here on presumed experimental proofs of multiwater paths (e.g., ref 44) from the observed kinetic isotope effect (KIE). Unfortunately there is no way to obtain a unique mechanistic proof form experimentally observed KIE since both the 1W and 2W paths are expected to have similar KIE (in fact the 1W is expected to have a larger KIE). Here it is essential to evaluate the KIE for both mechanisms before reaching any mechanistic conclusion (see the analysis of a related problems in ref 17). It is encouraging to note that a recent work[15] has obtained results that are very similar to our results, exploring the 1W mechanism and using similar search to the one used here (doing this however with a QM/MM model, but with a relatively poor sampling). However, that work did not explore the 2W mechanism.

It should be noted that, even if the path with a concerted PT through two water has slightly lower energy than that of PT from the nucleophilic water, it has little to do with the term "water as a base mechanism" proposed in ref 13. That is, water as a base mechanism requires that a water molecule would be the base in the reactant state. Now if the definition is extended to the TS then the water should be a real proton acceptor rather than serve as a shuttle where the phosphate remains the ultimate base and where the barrier is correlated with the pK_a of the phosphate oxygen. In fact, the issue of PT assisted by an additional group at the TS is more interesting, when one considers the GTP hydrolysis in Ras/GAP. In this case, it is possible that the mechanism with Gln61 serving as a proton shuttle in the TS has a similar energy to that of the direct PT from the attacking water. Of course, getting the enormous effect found in ref 24 is completely unrealistic, but some contribution from glutamine assisted PT in the TS is possible. Here we believe that the Gln 61 mainly acts in an allosteric mechanism as was found in our studies that simulated the structural changes associated with the mutation. In fact, it is even possible that TS with contributions from protonated (or partially protonated) Gln helps the allosteric transition. Interestingly, if a path with a partial PT to Gln stabilizes the TS, relative to the 1W path mechanism, we would expect the Gln61Ala mutation to let a penetration of an additional water and still keep a similar rate with the 2W mechanism (whereas the rate is reduced in this mutation by 6 orders of magnitude). Furthermore, we note that the idea of a PT to an additional group in the TS becomes more problematic in the related case of the activation of EF-Tu by the ribosome and the effect of mutating His84 on the activation,[39] where the structural changes are known experimentally.[47−54] In this case, which was explored in our study,[55] the His84 is already protonated in the ground state so it is hard to see the advantage of a path that does not involve the 1W mechanism. At any rate, any serious attempt to challenge the 1W mechanism in proteins must involve much more careful studies than those reported in previous attempts to promote the 2W and related paths. Here we believe that using the PD approach, with the EVB as a reference potential, provides the most promising option of quantifying the free energy difference between the 1W and alternative mechanisms.

One of the most instructive findings of the present work is the realization that the 2W is found spontaneously by approaches that just map R_2, whereas the finding of the 1W path (as well as the 2W least energy path) requires a more sophisticated 3D search and is likely to be missed by

oversimplified landscape search approaches when RC is composed of R_1 and R_2 only. This should serve as a guide and warning to those who are performing cutting-edge studies of complex biologically relevant systems. In fact, the above warning is relevant not only to energy minimization approaches but also to the far more sophisticated MTD approach (and to any other improved sampling strategies).

Studies of phosphate esters hydrolysis are another illustration of the importance of proper choice and representation of the RC. Here one should resist the temptation of reducing a chemical complex problem to a technical issue, which can be solved using a black box approach no matter how elegant it might seem (without fully examining, and controlling what the black box does). An example of this point seems to be given by the selection of the coordination number between all γ-oxygens and all water hydrogens as a collective variable in MTD simulations of ref 13. Such a coordinate is unlikely to give sufficient control since the constraining force acts on the particles in the narrow shell around the specified coordination radius[56] and there is a danger of getting some artifacts or discriminating one path over the other while being deceivingly ensured in exploring all of the possible options. In fact, there is no effective way to choose the correct collective variables (and correspondingly the auxiliary MD variable) for a chemical reaction without focusing on understanding the nature of the reaction surface (which is done in our strategy by mapping the solution surface). One should realize that the ability of MTD CV to automatically determine the reaction path is an assumption, which was tested only on simple cases,[57,58] and that the validity of this strategy needs to be repeatedly validated with the well-established conventional methods when moving to new systems. This is especially true when one deals with chemically challenging problems and may face the risk of reaching what may be a misleading conclusion.

In addition to our emphasis on performing a careful mapping while trying to reach mechanistic conclusions, we would like to emphasize that using incorrect (and sometimes misleading) terminology, such as calling a perfect assoc/conc path a dissociative path leads to unnecessary confusion in the field. For example although the schematic diagrams of ref 13 might look like dissociative paths, the actual simulations (i.e., Figure 4 in ref 13) barely bear any features of a dissociative path. Here we also would like to point out that a path through which we move to a given TS defines the mechanism much more rigorously than the position of the TS or the intermediate. Thus the intermediate (INT in Figure 3) could have been obtained with a dissociative path or an associative path, but in the work of refs 11 and 13, it was obtained through the associative path. Therefore the reaction should not be described as "dissociative" and clearly not as a metaphospate mechanism. Similar problems are associated with terming a case where a water molecule serves as a shuttle for the proton on the way to the phosphate as "water as a base mechanism", since this does not present a careful analysis of the validity of the phosphate as base mechanism (since the actual base is the phosphate).

In our view when one faces a complex case with several mechanistic alternatives the starting point should be exploration of the solution surface with careful systematic mapping along the key coordinates. After such a step one can move to more automated sampling methods.

In addition to the above works that were used to demonstrate our concerns, it is useful to comment on some other works from the rapidly increasing QM/MM studies of the hydrolysis of phosphate monoesters. One example, is a relatively careful CPMD study,[14] which explored the reaction in water. That is, these authors studied the hydrolysis of the highly charged reacting systems with only 54 water molecules over very few picoseconds and concluded that "The latter (dissociative reaction) has an activation energy of 35 kcal/mol, where 25 kcal/mol can be assigned to the P−O bond breaking and 10 kcal/mol to the artificial stability of PO_3^- resulting from the small size and the short time scale of the simulation. The path and energy barrier (39 kcal/mol) of the less-favorable associative reaction suggest that it is possible only under conditions where the lytic water is already deprotonated to OH^-". Of course, presuming what the effect of the missing solvent would be is not a fully valid scientific approach for examining alternative mechanisms. Furthermore, the authors state (perhaps, by not paying enough attention) that the "reactions discussed are very fast (9−13 ps)" overlooking the fact that the reaction has an activation energy that leads to reaction rates of months. Subsequent studies by the same authors[26] tried to explore ATP hydrolysis in actin using MD simulations with an MM force field to generate starting points for cluster modeling. In this case, a perfectly valid S_N2 TS is called "dissociative", and a reaction where another water serves as a base is defined as an "associative" mechanism (the same problem mentioned above). The authors suggest that their work discriminates between different mechanisms, while presuming that their model includes a significant part of the protein electrostatics. Unfortunately, this work overlooks the fact that sampling with an MM force field does not reflect the correct electrostatic coupling between the reactants and solvent and therefore cannot be effectively used in reliable QM free energy calculations of charge separation and transfer processes.

Another example is the recent study of the hydrolysis of ATP in solution,[59] which presented an example of a brute force sampling. Unfortunately, this work has not provided a clear 2D free energy surface and has not attempted to look at the free energy for PT or to control the corresponding path. In fact, the study of the associative path has not explored the corresponding PT barrier, whereas the study of the dissociative path (where the PT may have occurred spontaneously), presents another serious problem. That is, the mapping in this case only involves R_1 and the TS looks as an associative TS based on Figure 4 of ref 59 (no other information is given). In this case one may wonder whether the assertion of the authors that the water attack occurs only at the TS, is correct. That is, because R_2 was not monitored we only have one snapshot with a large R_2 but no real relevant statistics. However, studies that properly constrained R_2 and R_1 have reached very different conclusions. Here what is needed is a clear 2D PMF. In fact one may take significant exception to the presumption that until ref 59 there was no work that "captured by simulations the relaxation dynamics and its role in catalysis". These aspects have, of course, been captured consistently and effectively in the EVB studies of ref 60 and in countless related studies of GTP hydrolysis (e.g., ref 55). Thus the challenge here is not the relatively trivial task of capturing the solvation effects (which can be done even with ab initio QM/MM by the approach of ref 61) but the reliable evolution of the PMF along the key paths. Another interesting recent work that presumed a 2W type path is the work of ref 62, which studied the reaction in Ras (without being aware of any previous serious theoretical study). This work used a semiempirical QM/MM method, drastically overestimated the relevant barriers, and never

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explored the 1W path. More seriously, the calculations were only done for the PT path with the reactant state R_1 and R_2 and thus are irrelevant to the bond breaking process. An additional interesting and potentially useful recent study[63] reported energy minimization exploration of the reaction in F1-ATPase. Here again it was only TS2 that was considered, with only the 2W (3W) mechanism, overlooking the energetic of competing paths. The authors also tried to invoke a presumed consistency with the observed isotope effect, overlooking the fact that both 1W and 2W PT paths will have an isotope effect and that the same will happen in the associative and dissociative (1W) TSs.

Another interesting recent work which explored the 1W and 2W mechanisms in phosphate triester hydrolysis is the study in ref 64. This work concluded that the 1W associative barrier is 15 kcal/mol higher that the 2W (although paper has not provided the TS structure for the 1W path nor examined the paths with the concerted TS2 path). It is also of some concern that this work overlooked early studies that examined the problems with mixed explicit and implicit models.[65] That is, the authors of ref 64 seem to misinterpret the emphasis of ref 65 regarding the problems which arise for implicit models which include non reacting explicit water molecules as a recommendation for not including the explicit water molecules involved in chemical reaction. In fact, ref 65 stresses the importance of exploring the resulting multiple local minima and the entropy associated with the additional degrees of freedom, and never suggested to exclude important solvent molecules that are involved directly in the chemistry. At any rate, even if the barrier of the 1W path is actually higher than that of the 2W barrier, we note that in the triester case we found that the pK_a gradient that facilitated the reduction of the 1W barrier in the monoester case (see Figure 10) does not exist. In fact, the pK_a of the accepting oxygen is reduced strongly upon moving from the monoester to the diester and then to the triester.

The same points considered here (namely, the study of phosphate hydrolysis in water) are relevant to phosphate hydrolysis in proteins. Here the chance of getting problematic results is much larger and the confusion is likely to persist for a longer time. This is why we view EVB studies as the most reliable *current* approach for exploring the reaction in enzymes. This means, however, that one must start with a very careful search of all key mechanistic options in solution and then ask (without prior bias) what happens to these mechanisms when we move to the protein. Of course, with increasing computer power we can start to explore the effect of moving from the EVB to QM(ai)/MM surfaces by the PD approach. However, this approach is likely to give reasonable results only with very extensive EVB sampling.

We would like to emphasize that the present study found similar activation free energies for the 1W and 2W paths, but more careful PD studies would be absolutely needed to establish which (if any) of the paths is the rate limiting. It is important to note in this respect that the current calculations have not consider the entropic effect that is likely to increase the barrier for the 2W path relative to that of the 1W path by 2−5 kcal/mol (a careful determination will require the use our restraint release approach as described in ref 66). Furthermore, it would also be important to consider the difference in quantum mechanical tunneling effects that are likely to reduce the 1W barrier more than the 2W barrier (where we can use our QCP approach[67]). On the other hand, having Mg^{2+} ion is likely to reduce the pK_a of the phosphate oxygen and thus to favor the 2W path.

It is also crucial to remind the readers that the main arguments in the field of phosphate ester hydrolysis has been about the associative versus dissociative mechanistic pathways. Key experimental workers (see discussion in ref 17) presumed that there are experimental evidences that the hydrolysis of phosphate ester bond in solution (and thus also in proteins) precedes through a dissociative pathway, whereas we have shown (e.g. ref 17) that no such evidence exists (all observations are consistent with both mechanisms) and that in most cases we have associative or concerted paths. Here the careless support of the dissociative mechanism by computational workers, who actually obtained associative mechanism, has been one of the main motivations for writing this paper. That is, the issue is much more that naming a mechanism by an incorrect name, since doing so allows parties in this major scientific controversy to exploit incorrect assertion, from otherwise reasonable calculations.

■ ASSOCIATED CONTENT

Supporting Information

The data used to generate the free energy surfaces presented in Figures 7 and 8 are presented. Also, the Cartesian coordinates for the critical points along the surfaces of 1W and 2W pathways are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: warshel@usc.edu.

Notes

The authors declare no competing financial interest.

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