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On the Mechanism of Hydrolysis of Phosphate Monoesters Dianions in Solutions and Proteins

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Abstract

The nature of the hydrolysis of phosphate monoester dianions in solutions and in proteins is a problem of a significant current interest. The present work explores this problem by systematic calculations of the landscape of the potential surfaces of the reactions of a series of phosphate monoesters with different leaving groups. These calculations involve computational studies ranging from ab initio calculations with implicit solvent models to ab initio QM/MM free energy calculations. The calculations reproduce the observed linear free energy relationship (LFER) for the solution reaction and thus are consistent with the overall experimental trend and can be used to explore the nature of the transition state (TS) region, which is not accessible to direct experimental studies. It is found that the potential surface for the associative and dissociative paths is very flat and that the relative height of the associative and dissociative TS is different in different systems. In general, the character of the TS changes from associative to dissociative upon decrease in the pK_a of the leaving group. It is also demonstrated that traditional experimental markers such as isotope effects and the LFER slope cannot be used in a conclusive way to distinguish between the two classes of transition states. In addition it is found that the effective charges of the TS do not follow the previously assumed simple rule. Armed with that experience we explore the free energy surface for the GTPase reaction of the RasGap system. In this case it is found that the surface is flat but that the lowest TS is associative. The present study indicates that the nature of the potential surfaces for the phosphoryl transfer reactions in solution and proteins is quite complicated and cannot be determined in a conclusive way without the use of careful theoretical studies that should of course, reproduce the available experimental information.

I. Introduction

Many biological molecules contain phosphate and the reactions that break, connect or exchange the bonding between such groups play a major role in key processes ranging from the replication of the genetic material to energy and signal transduction (for reviews see for example 1-7). One of the most

important reactions in such systems is the hydrolysis of phosphate monoesters such as GTP and ATP that play a major role in energy and signal transduction processes. These systems have been explored extensively in the past by different experimental and theoretical approaches that examined factors that can change or modulate the rate relevant constant (e.g. 1, 3, 5, 8-25). Furthermore, significant insight has been obtained from studies of the mechanism of the corresponding hydrolysis reactions in solution (e.g. 2, 6, 26-33). Additional insight has been provided by studies of related systems (e.g. 34-38). Nevertheless, at present we still have a limited understanding of the detailed reaction paths in solution and in enzyme active sites. The problem stems to a large extent from the fact that the energies of the transition states of phosphate hydrolysis reactions are relatively high, and it is very hard to characterize such transition states experimentally via key intermediates. Thus, despite frequent implications that many of the mechanistic issues have been resolved experimentally (e.g. refs 39,40), it is extremely hard to deduce the nature of the transition state (TS) in a unique way from the available experiments. For example, a careful analysis[8, 41] has indicated that the difference between associative and dissociative paths cannot be deduced from the observed LFER or the observed isotope effect, as well as from other traditional markers. In view of the difficulty of obtaining a unique mechanistic insight by direct experimental studies, it is important to try to exploit computational approaches in studies of the hydrolysis of phosphate monoesters. It is crucial, however, to demand that the computational analysis will reproduce all key experimental findings.

Since our initial systematic evaluation of the surfaces for associative and dissociative phosphate hydrolysis[34] in solution, there were several attempts to explore this crucial problem. We will not consider here gas phase studies that were reviewed in ref. 37, since these studies cannot be used to resolve mechanistic problems in condensed phases. Here we would like to clarify that gas phase calculations that focused on the existence of a pentacoordinated intermediate with a clear minimum on the potential surface are not particularly useful. That is, this issue might be relevant in some solution reactions but is of a little significance when one considers gas phase calculations with say 80 kcal/mol barriers where the possible difference between having a minimum of a few kcal/mol and not having it is questionable.

Some studies yielded interesting insights in the reaction of monoanions as was done for instance in ref. 36, which overlooked however the entropy cost associated with water bridge dissociative states (see discussion in ref. 9). While this issue is of a significant interest our focus here is on the studies of the hydrolysis of phosphate dianions. In this respect it is important to clarify that the controversy about the nature of the TS (associative or dissociative) is arguably the most important open mechanistic problem in the field. Thus attempts to imply that such controversy does not exists at least in the case of diesters[42] and that all workers support the associative mechanism are not justified. In fact even the associative nature of the hydrolysis of phosphodiesters has not been established or widely accepted (e.g. for a recent review see 43).

Recently reported studies vary in their sophistication and the relevance to the key issue of the energetics of the associative and dissociative path. For example the systematic study of Wang et al.[33] examined the associative path of the dianion of mono methyl phosphate but focused on the monoanion dissociative path that was then discussed as a support for a general dissociative mechanism (although the reaction in Ras involves probably a dianion system). A recent Car-Parrinello DFT study of Akola and Jones[44] found similar energy barriers of about 40 kcal/mol for the dissociative and associative paths of ATP with magnesium ion in solution but argued, without sufficient justifications, that the calculations for the dissociative barrier must represent a major overestimate. These workers also provide estimates for the reaction free energy but the corresponding PMF seems to reflect a far too short sampling time. A systematic QM/MM study[32] of the hydrolysis of triphosphate in solution was reported recently. This study was instrumental in identifying an associative mechanism with a late proton transfer. However the calculated barrier was about 20 kcal/mol rather than the 27 kcal/mol barrier that is observed for such a system.

Although the above studies were quite instructive we feel that it is essential to generate a more quantitative picture that will reproduce the overall experimental trend in a series with different leaving groups. It is also crucial to evaluate the complete free energy surface rather than only isolated transition states in order to understand the overall nature of the associative or dissociative reaction paths. The present work will focus on the analysis of the reaction surface of the hydrolysis of phosphate monesters dianions and related systems, using state of the art computational approaches. This study can be considered as a follow up to the earlier attempt to map theoretically the associative and dissociative potential energy surface (PES) in solution[34], with a focus on the availability of more computer power and several alternative approaches. We will also exploit the recent availability of more specific experimental information about the magnitude of the activation barriers of the hydrolysis in key systems (e.g. 45-48).

It will be shown that our free energy surfaces reproduce the observed experimental trend over a wide range of rate constants and basically reproduce the experimental LFER. The experimental validation of the calculated surfaces will be taken as an indication that the calculated surfaces and reaction paths are reasonable. It will be shown that the lowest TS changes gradually from associative to dissociative upon increase in the acidity of the leaving group. We will also explore the effect of a magnesium ion on the solution surface and then move to the Mg^{2+} catalyzed RasGAP reaction. Here we will argue that the very flat TS region of the solution reaction can be easily changed by the protein active site and that the TS in the RasGAP reaction involves an associative TS.

II. Defining the problem

The transition states of phosphate hydrolysis reactions have been traditionally classified (e.g. ref 30, 49) as associative or dissociative transition states according to the distance between the reacting phosphate and the leaving group, R_1, and the distance to the attacking nucleophile, R_2 (see Fig. 1). This definition is related to the More O'Ferrall-Jencks (MOFJ) diagrams[50, 51] but it is drawn in terms of bond lengths rather than the bond order description used e.g. by Williams[49].

The definition of Fig.1 is sometimes extended to "loose" TS[49], but the main features are uniquely defined in terms of the 2 dimensional surface outlined in figure 1. Here it is crucial to state that the overall surface and reaction path provide a more unique definition than alternative attempts to clarify the hydrolysis mechanism in terms of the sum of distances (e.g. ref 52) or other structural features. It is also important to note that the conventional definition in terms of bond order does not provide any clear insight about the energetics on the reaction surface, nor information about the charge distribution (e.g. see ref. 30). More important is (as will be clarified below) that definitions in terms of the observed LFER and the corresponding effective charges are quite problematic.

It is also useful to clarify that the proton of the attacking water is always allowed to find its lowest energy at any point on the map. Thus the position of the proton is not a part of the definition of the associative or dissociative path (although it is of course an interesting parameter). With this in mind, it is not so productive to include the proton position in the definitions of the path (e.g. ref 16). Similarly, defining the substrate assisted mechanism as a distinguishable path (e.g. ref 16) is problematic, since even when the proton transfer from the attacking water to the phosphate oxygen atoms is concerted with the nucleophile attack (e.g. ref 9, 34) we still have a substrate as a base mechanism. At any rate, in view of the above points we believe that the role of theoretical analysis of phosphate hydrolysis is to provide a clear description of the landscape of the reacting system and to locate key reaction paths and key transition states. It is also important to examine whether various experiments can actually help in discriminating between different assumed mechanistic options or, more likely, that the experimental information is less unique than what has been commonly assumed. The present work will address the above problems by evaluating free energy surfaces of representative systems and exploring the balance

between associative and dissociative pathways. The conceptual part of the analysis will be guided by the empirical valance bond (EVB) description[53-55]. This description basically considers the four corners of Fig. 1 as the centers of parabolic surfaces (see Fig. 2), that correspond to different zero order diabatic states. The mixing of these surfaces by the EVB off-diagonal terms leads to the actual adiabatic reaction surface. If the surface follows more or less one of the extreme paths (associative or dissociative) it is easy to quantify the nature of the observed LFER by shifting the parabolas in the lower part of Fig. 2, as was done in ref. 8; the inverse is not true and the observed LFER cannot tell us about the nature of the parabolas, since the shifts of the parabolas in the different cases can lead to a similar LFER, (see ref. 8 and the present work).

III. Methods

This work tries to generate "consensus" surfaces for phosphate hydrolysis in solution by combining the results of several methods in order to establish the error range of the calculated results, and by insisting that the calculated barriers will be consistent with the corresponding observed rate constants.

To generate the effective free energy surfaces (PES) for the hydrolysis reaction we started by constraining the values of the two chosen reaction coordinates, R_1 and R_2 (see Fig. 1) and optimizing the structures of the constrained system in the gas phase (note that we later map the surface in solution) using the density functional theory (DFT) based method B3LYP/6-31+G* . The resulting structures are subsequently solvated using the COSMO continuum model[56, 57] and verified in some cases using the Langevin dipoles (LD)[58, 59] model in its standard implementation. An additional local relaxation of the energy of the given solvent model for points (R_1, R_2) on the reaction surface of the solvated system is also applied in cases where we are at a TS region. The error range associated with the choice of the quantum method and the basis set is also established. This is done by evaluating selective points using the methods MP2/6-311++G** and B3LYP/Lanl2DZ. The evaluation of the corresponding PESs required us to address two problems. The first problem is the dependence of the optimized structures on their initialization. This problem is handled by starting the optimization procedure from several initial structures for a given point (R_1, R_2), and taking the lowest value as the corresponding effective energy. The second problem arises from the neglect of additional possible reaction coordinates, in our case the coordinate, X, that describes the proton transfer from the attacking water to the phosphate oxygen atoms, (Fig. 1). This problem is handled by examining the structures along the reaction paths carefully, after finding the initial reaction paths on our PES, to ensure that all reaction paths develop continuously, i.e. that all points on the PES are actually connected. In cases where we find discontinuities, especially for the proton transfer reaction coordinate, we estimate the energy barrier for the proton transfer with additional energy scans in the direction of this coordinate. At any rate, at the end of our mapping procedure we end up with a carefully scanned solution surface.

While the accuracy of the simplified solvent models mentioned above have been found to be reasonable in earlier studies, it is important to examine the possible effect of moving to a more atomistic solvent model. This is done here by using a special combined ab initio quantum mechanics molecular mechanics free energy perturbation treatment (this approach is referred to here as a QM(ai)/MM-FEP approach). In general we advocate the evaluation of the free energy surface of reactions in solution by using a QM(ai)/MM approach that uses the EVB as a reference potential[60]. This approach is particularly effective when one allows the solute coordinates to change during the mapping process. Here, however, we restrict ourselves to a simpler approach where the solute coordinates are fixed at each point (the

missing entropic effects will be considered below). In this case we are interested primarily in the solvation of the fixed solute and we can use the relationship:

$$\Delta G(R_1,R_2) = \Delta E_{gas}\left(R_1,R_2\right) + \Delta G_{solv}\left(R_1,R_2,\mathbf{Q}_S^g\right) + \Delta\Delta G_{pol}^S(\mathbf{Q}_S^g \rightarrow \mathbf{Q}_S^s) \qquad (1)$$

Where $\mathbf{Q}_S^g$ and $\mathbf{Q}_S^s$ are the vectors of the partial charges of the solute in the gas phase and in solution (when the solvent potential is included in the ab-initio Hamiltonian). These partial charges are taken as the charges derived from the electrostatic potential (ESP) according to the Merz-Singh-Kollman scheme[61, 62]. $\Delta\Delta G_{pol}^S$ is the solute energy upon change of its electron density from gas phase to its solution value. The solvation free energy term $\Delta G_{solv}\left(R_1,R_2,\mathbf{Q}_S^s\right)$ is evaluated by using a standard adiabatic charging (AC) free energy perturbation (FEP) procedure (e.g. refs 53, 63), changing the solute residual charges from zero to their value in the gas phase in n+1 integration steps using:

$$\mathbf{Q}_m = \mathbf{Q}_S^s \lambda_m \qquad (2)$$

and representing the mapping potential by

$$U_m = U(\mathbf{Q}_m)_{Ss} + U_{ss} \qquad (3)$$

where U_{Ss} and U_{ss} are the solute-solvent and solvent-solvent potentials parts of the potential surface as represented by the ENZYMIX force field. With this mapping potential we use the standard FEP equation

$$\Delta\Delta G_{solv}(\lambda_m \rightarrow \lambda_{m+1}) = -\beta^{-1} \ln < \exp(\ -(U_{m+1} - U_m)/RT)\ ) >_{U_m}$$

$$\Delta G_{solv}\left(R_1,R_2,\mathbf{Q}_S^g\right) = \sum_{m=1}^{n+1} \Delta\Delta G_{solv}(\lambda_m \rightarrow \lambda_{m+1}) \qquad (4)$$

where $\beta = (1/k_B T)$, k_B is the Boltzmann constant and T is the absolute temperature. In this QM(ai)/MM FEP approach we fix the solute charges , $\mathbf{Q}_S^g$. Thus the effect of polarizing the solute (the last term in eq.1) is only evaluated in a mean field using the LD solvent model as discussed in ref. 64. We also have an alternative approach where

$$\Delta G(R_1,R_2) = \Delta E_{gas}(R_1,R_2) + \Delta G_{solv}\left(R_1,R_2,\mathbf{Q}_S^s(r^s)\right) \qquad (5)$$

where now the potential U_m fluctuates upon fluctuations of $\mathbf{Q}_S^s$ as a result of the solvent fluctuations. Our preliminary studies indicate that both approaches give similar results due to the reasonable physical picture provided by the last term of Eq.1.

In principle we should include in Eq.1 zero point energy effects and activation entropies. However, the zero point contributions to the activation barriers were found to be smaller than 1 kcal/mol (see section IV-b). The activation entropy effect was found to be much smaller than their gas phase barrier estimates and was neglected (see discussion in 65). Furthermore the experimental studies of Wolfenden[48] established that for the reactions of the type studied here (hydrolysis by water) the contributions of the activation entropies are small. A more careful study that will use a restraint release approach[66] is left to subsequent studies.

The QM(ai)/MM calculations were implemented as described in details in ref 67. Our treatment used standard link atom technique rather than the more reliable hybrid orbital type treatments (e.g. 58, 68) because we found it very convenient to link our MM program MOLARIS[69, 70] to standard QM programs (in this case GAUSSIAN 98[71]) through an automated external script. The calculations were performed with the B3LYP/DFT method with 6-31+G* basis set for the QM/MM calculations.

The specific FEP parameters used in this work are the standard ENZYMIX parameters and the simulations were performed using the MOLARIS program with a surface constraint all atom solvent (SCAAS)[72] spherical boundary conditions of 22 Å radius and the LRF long range treatment[73]. The solvation QM(ai)/MM free energies were evaluated by performing the AC-FEP calculations[63] with 31 windows of 10ps length each and a time step of 1fs. The simulations were performed at 300K.

IV. Results and discussion

a) Free energy surfaces for the hydrolysis of phosphatemonoesters in solution

(i) Monomethyl phosphate dianion

We started our systematic study by evaluating the free energy surfaces for the monomethyl phosphate dianion. The results of the calculation are summarized in Fig. 3 and Fig. 4 as well as in Table 1 of the supplementary material. This system was explored systematically in our previous study[34] and the present results are similar to those obtained before, including the results considered briefly in the study of ref. 33, that focused, however, on the monoanion. Ref 34 gave two associative TSs of 40 kcal/mol and 43 kcal/mol and a dissociative TS of 56 kcal/mol, while ref 33 gave an associative barrier of 42 kcal/mol for the dianion.

As seen from the figures and the table the hydrolysis reaction is associative with a relatively high barrier of 47 kcal/mol. This result is in a good agreement with the experimental results[46] for alkyl phosphates (about 44 kcal/mol for methylphosphate at 25 C°). Interestingly the calculated activation barrier for hydrolysis of methylphosphate by OH^- is about 40 kcal/mol. Thus it would be interesting to assess the contribution from this OH^- pathway to the hydrolysis of monomethyl phosphate in water and to examine whether the experimental estimate of the contribution to the barrier from the hydrolysis by water should be slightly modified. The nature of the overall surface can be described by case A in Fig. 2.

(ii) Phenylphosphate dianion

Next we evaluate the surface for a case that involves a leaving group with a lower pK_a, namely the phenyl phosphate dianion. The results of these calculations are summarized in Fig.5 and Table 2 of the supplementary material. As seen from the figure we still have as associative TS but the activation barrier is much smaller than in the case of the monomethyl phosphate. The calculated barrier of 35 kcal/mol is again in very good agreement with the observed value of about 35 kcal/mol[46].

(iii) Mono methyl pyrophosphate trianion

After establishing the strong dependence of the barrier on the pK_a of the leaving group we returned to the systems that are more relevant to the hydrolysis of GTP and ATP (although in this systems the electrostatic repulsion is screened by a Mg^{2+} ion) . As a representative system we considered cases with methyl phosphates as a leaving group, since the effect of the α phosphate group on the hydrolysis of the γ-β bond is relatively small[8, 19]. We start the study of these systems by evaluating the surface for the hydrolysis of the mono methyl pyrophosphate trianion. The results of the calculations are summarized in Fig.6 and Fig.7 as well as in Table 3 of the supplementary material. As seen from Fig. 6 the dissociative TS is now at a lower energy than the associative TS. Thus we conclude that the decrease in the pK_a of the leaving group changes the character of the TS from associative to dissociative. The EVB behaviour of the surface in the path through the dissociative TS can be described by case B of Fig. 2. The calculated barrier is about 34 kcal/mol which is in a very good agreement with the observed barrier for the pyrophosphate trianion (about 33 kcal/mol)[48]. The success of our calculations of this highly charged system indicates that the strong electrostatic repulsion is properly screened by the solvent model used. It

is also apparent that the real solvent (i.e. water) screens theses interaction quite effectively since as will be shown below the trianion system falls nicely of the observed LFER.

(iv) Mono methyl pyrophosphate dianion

In view of the relatively high barrier of the trianion system it is important to consider the effect of protonation of the pyrophosphate system. This is done in the calculations summarized in Fig. 8 and Table 4 of the supplementary materials. Now the difference in energy between the associative and the dissociative TS becomes quite small. Although the protonated system has a lower barrier than the deprotonated system it is very likely that the hydrolysis of GTP and ATP in proteins involves deprotonated systems. It is interesting to note that in the reactant state the proton is always transferred to the phosphate that is hydrolyzed even when the position of the proton is initialized at the leaving group. This proton is eventually transferred back to the leaving group as expected on the reaction path toward the product state.

(v) Magnesium mono methyl pyrophosphate trianion

Despite our success in reproducing the barrier for the hydrolysis of mono methyl pyroposphate trianion, we felt that a good reference system for the GTPase and ATPase reactions in proteins can be provided by the hydrolysis of mono methyl pyrophosphate in the presence of an Mg^{2+} ion. The position of the magnesium ion was initialized between the two phosphates to form a bidentate complex (see Fig. 10), resembling the usual coordination of the magnesium ion with GTP or ATP in enzymes like Ras p21 or ATPases. Furthermore we included into the QM system four additional water molecules around the magnesium ion to complete its first solvation shell. The results of these calculations are summarized in Fig. 9, Fig. 10 and Table 5 of the supplementary results. This solution system has basically three TSs with similar energy but the dissociative TS is likely to be with higher energy in active sites of proteins. That is, although it is absolutely essential to examine the situation inside the protein by QM/MM calculations that include the protein (as is done in section IV) it seem from the surface of Fig 9 that moving to the dissociative TS, which involves a large increase in the distance between the neuclophile and leaving group, costs only a few kcal/mol. Thus even small steric forces in an active site that is originally complementary to the reactant state are likely confine the reaction surface to the associative region where the distance between the nucleophile and the leaving group is small. At any rate, the calculations indicate that the potential surface involves a flat ridge at the TS region and that small perturbations can easily shift the TS position. The surface of Fig. 9 is quite complicated and can be considered as a combination of case A, B and C in Fig. 2. This can be represented with a single EVB surface with special mixing terms. Interestingly the lowest activation barrier for the associative path is around 32 kcal/mol as compared to the observed barrier of 28 kcal/mol[74].

(vi) Other systems

In addition to the systems considered above we also examined additional systems to gain more insight about the overall trend in phosphate hydrolysis reactions. The additional systems include the hydrolysis of the acetyl phosphate dianion, with a very good acetate leaving group. The corresponding results are included in our LFER considerations that will be described below.

b) Examining the sensitivity of some experimental observations to the nature of the TS

The present study allows us to explore the validity of the long standing assumption that the observed LFER can be used to determine the nature of the TS in phosphate hydrolysis reactions (e.g see ref 39). Our previous studies[8, 41] already clarified that the traditional assumptions about the relationship between the observed LFER and the nature of the TS are very problematic. The main problem is the LFER should be based on at least three VB states (e.g. Fig. 2) and thus does not follow the traditional assumptions in physical organic chemistry. This point has also been demonstrated in a pioneering ab initio study of phosphate monoesther dianions in solution[41], that involved methanol as a nucleophile and substituted phenyl leaving groups. Here we continue along the previous line of reasoning by comparing the calculated and observed LFER for the hydrolysis of different phosphomonoesters. The results of the present study are summarized in Fig. 11 and show that we could reproduce the observed trend over a very wide range of rate constants. Apparently as pointed out above, the rate determining TSs are very different for the different molecules considered in the LFER study. Thus it seems clear that the fact that a given molecule that follows this LFER cannot be used to deduce the nature of the corresponding TS.

The LFER observed in phosphate hydrolysis reactions has been traditionally used to define effective TS charges (e.g. refs 75, 76). This definition relates the change in the charge of the leaving group to the Brønsted α or to the ratio between β_{lg} and β_{eq}[76]. Such an analysis is very reasonable and would be physically consistent in the case of intersection of two VB states. Otherwise it may turn out to be just a way for classifying the given reaction. Here we perform a preliminary analysis of the nature of the effective charges by calculating the change in the charges of the leaving group from the reactant to the product state for the associative and dissociative paths of the hydrolysis of the mono methyl pyrophosphate trianion. The corresponding results are summarized in Table 3. The first point that emerges from the table is the fact that the change in the TS charge upon moving from the RS to the TS does not follow a simple rule. For example when we have CH_3O^- as a leaving group the changes are -0.22 and -0.68 for the associative and dissociative TSs, respectively, while for the case with $PO_4CH_3^-$ as a leaving group the changes are found to be -0.30 and -0.45 for the associative and dissociative TSs respectively. This means that the effective TS charges cannot be used to describe the different mechanisms. Similarly and quite significantly it indicates (in contrast to the implications of ref. 39) that the electrostatic interaction of the environment in Ras and related systems can stabilize the associative and dissociative TSs in a similar way[9]. This point will be quantified below. The second point is that the change in ab initio charges between the RS and TS are not similar to the changes in charge deduced by traditional physical organic chemistry (e.g. refs 75-77). For example in the case of ATP hydrolysis[39] and GTP hydrolysis[78] in solution it is assumed that the change in charge is -0.83. On the other hand, our ab initio calculations give -0.30 for the low energy TS and -0.45 for the dissociative TS. The situation becomes even more striking in the case of the hydrolysis reaction in RasGAP when the changes in charge are -0.28 and -0.32 for the associative and dissociative TSs, respectively. Here one could argue that the effective charges should not be equal to the ab initio charges but the problem is, in fact, more fundamental. That is, traditional physical organic chemistry approaches provide an excellent estimate of the effective charge in cases of two intersecting energy parabolas, which might be a reasonable description in the concerted case (case C in Fig. 2). However, in the more general case the most unique definition of group charge can probably be obtained by the VB approach that provide the natural definition of the degree of charge transfer (see e.g. ref. 53, chapter 2) and can quantify the effect of the environment and various substantiates on such charges (see also ref. 79). Now the charge changes obtained by well calibrated EVB Hamiltonians should be equal to the corresponding ab initio ESP charge changes, since this is the way we calibrate the EVB surface. This means that the ESP charges provide what is probably the best description of the effective charges and that our conclusions about the complex nature of these charges are valid.

The O16/O18 isotope effect has been used extensively in attempts to determine the nature of the transition state in phosphoryl transfer reactions (e.g. refs 2, 16). Although it is tempting to accept the

assignments obtained from isotope effect studies it has been argued that the corresponding analysis is far less unique than what has been commonly assumed[8]. Basically the assumption of the relationship between the isotope effect and the reaction path are in some respects circular since the nature of the TSs have never been established by a direct experimental approach and the basic arguments about the expected isotope effect are problematic[8]. The general difficulty of obtaining unique mechanistic interpretation of isotope effects experiments has also been noted by the experimental community (e.g. 80). Interestingly, a recent theoretical study[81] of transferification found what is basically an associative TS with isotope effects larger than one that could have been probably interpreted traditionally as an evidence for a dissociative mechanism.

In view of the above problems we calculated the isotope effects for the hydrolysis of the magnesium mono methyl pyrophosphate trianion (shown in Fig. 9), considering different TSs as the rate limiting states. The calculations were done using the B3LYP/6-311++G method for the gas phase TSs (described in the supplementary information), with the isotopic composition described in Fig. 12. The calculated zero point energies are summarized in Table 4. These zero point energies were used to evaluate the corresponding isotope effects, which are summarized in Table 5. The calculations indicate that the isotope effect expected from associative and disociative TSs is quite similar and thus cannot be used to establish the nature of the TS.

Interestingly, the calculated isotope effects are in the range found experimentally for the GTPase reaction of Ras[16] which are 1.0013 for $\gamma^{18}O_3/\gamma^{16}O_3$ and 1.0128 for $\gamma^{18}O_4/\gamma^{16}O_4$, respectively. More accurate results could have been obtained by evaluating the isotope effects in solutions and proteins using the centroid path integral methods[82, 83] and in particular the robust quantized classical path (QCP) simulation approach[84]. However, the required evaluation of realistic vibrational spectra in solution or proteins is quite demanding (for an effective approach see e.g. ref 20) and is left to subsequent works since our main aim is to establish the fact that the very qualitative reasoning that has been used in analyzing phosphate hydrolysis reactions is not supported by actual quantum mechanical calculations. Of course, it would be interesting to use more careful calculations to try to see which TSs in, for example, the reaction of Ras give a better agreement with the observed isotope effect, but such a study cannot be done by the current use of circular arguments and should be based on extremely careful simulation studies.

c) The free energy surface for the reaction of Ras

Armed with the insight from the above solution studies we can explore related enzymatic reactions. In particular we are interested in understanding the GTPase reaction of the RasGAP system. This GTP hydrolysis involves a large catalytic effect (about 11 kcal/mol) that has been the central subject of biochemical and structural studies of signal transduction (for reviews see 1, 85). Although significant progress has been made in the theoretical description of this reaction it has been hard to asses whether the reaction involves an associative or dissociative mechanism[1, 9, 15, 16, 85, 86]. The main difficulties have been associated with the fact that both pathways can be catalyzed by the protein in a similar way[9]. Here we perform a careful and systematic QM/MM study of the surface for GTP hydrolysis in Ras/GAP. The calculations started with an extensive relaxation using EVB potential surfaces similar to those used in ref 10. The relaxed structures were used as a starting point for the QM(ai)/MM FEP calculations that were done for the R_1 and R_2 space. Note that in each point of this surface we perform extensive FEP simulations that allow for a full relaxation of the protein environment. The results of this simulation study are summarized in Fig. 13 and Fig.14. Apparently the shape of the surface is similar to that obtained for magnesium mono methyl pyrophosphate trianion in water, but it seems to have more associative character. The calculated barrier height is found to be 14 kcal/mol, which is quite similar to the 16 kcal/mol observed barrier. The very good agreement may be somewhat coincidental since the calculations did not involve an actual evaluation of the potential of mean force (PMF). However, the

mechanistic conclusions are probably valid. A completely conclusive study might require additional exploration of the effect of different mutations and the evaluation of the free energy surface by an approach that also consider the solute entropy[60]. It would also be interesting to reproduce the observed LFER in Ras[15]. However, the mechanistic picture that emerges from the present study is probably significantly more conclusive than the assumed mechanistic information that emerged from interpretation of experimental results, such as isotope effects[16] or even from structural studies of transition state analogues (whose direct relevance to the exact transition state is hard to establish)
Finally it should be kept in mind, that the premise of several experimental attempts to explore the nature of the TS in Ras is very problematic. That is, it has been assumed in several recent works (e.g. refs 78, 87) that the transition state of the reference reaction in solution is dissociative and thus presumably the same is true for the TS in Ras. Apparently as shown in this work this assumption is not justified. That is, ref 39 envisioned a surface with an enormous barrier for the dissociative path (Fig 1 of ref 39),yet the careful study reported here indicates that all the experimental problems that were used to support the proposal of a dissociative TS are equally valid for supporting the proposal of a associative TS.

d) Examining the error range of the calculations

At this point it might be useful to consider the corresponding error range of our calculations and it s impact on our overall conclusions. We start by comparing in Table 1 the energetics of different key points on the surfaces of the magnesium mono methyl pyrophosphate trianion obtained by both the LD and the QM/MM –FEP approach of Eq 1-4. As seen from the table the very different models give similar results with deviations between 1 to 5 kcal/mol. The effect of using different quantum mechanical Hamiltonians was estimated to be relatively small since the B3LYP/6-311++G** method used here is quite reliable. Finally, we examined the magnitude of the zero point energy corrections (see Table 4) and found them to be smaller than 2 kcal/mol for the energy barrier in the most extreme case and smaller than 1 kcal/mol in most cases. We did not include the gas phase entropic contributions since our earlier studies (e.g. ref 65) indicated that these contributions are small and cannot be deduced from the gas phase calculations. Furthermore the experimental studies of Wolfenden[48] established that for the reactions of the type studied here (hydrolysis by water molecule) the contributions of the activation entropies are small .A more careful study that will use a restraint release approach[66] is out of the scope of this work.
It is important to reemphasize that our calculations are *not* based on taking gas phase transition states and solvating them but on a very systematic construction of the free energy surface for the solution reaction by a careful interpolation of values obtained by consistent calculations of the energies of the solvated quantum system, this includes a relaxation for R_1 and R_2 near the suspected TS, see for example discussion in the method section. Having the complete surface is also much more informative than having only some specific TSs, since it provides crucial information about the nature of the overall landscape. This should allow one to better appreciate the nature of the reaction paths. It is also important to point out that our conclusions on the rather late proton transfer step were determined by a thorough scanning and not by gas phase calculations and these results were contained by the different approaches used here. In this respect we like to emphasize that the possibility of proton transfer from the attacking water molecule at the TS of the reaction was also examined by careful and complete mapping.

Another point of concern is the ability of the present study to reproduce the proper screening for the strongly interacting negative charges in say the hydrolysis of the mono methyl pyrophosphate. Here we would like to clarify that we are not dealing with gas phase calculations but with proper calculations in solutions. In particular we are encouraged by obtaining similar results for the simplified solvent models

(The COSMO and LD models) and the microscopic QM/MM calculations. Furthermore it is important to point out that we have reproduced the actual experimental trend in such systems.

At this stage it is important to examine the relations between the calculations of the reference reactions and the corresponding observed results. As established in Table 2 and Fig. 11 we obtained a very encouraging agreement between the calculated and observed barriers where the calculated rate constant follows nicely the observed LFER. This lends significant credibility to the calculated results.

One might still argue that with an estimated error range of 2-4 kcal mole there is no reason to accept our conclusions. However, our main point is not the exact height of the associative and dissociative barriers but the fact that they are frequently similar and that their relative height changes with the pK_a of the leaving group. In our view this point has been established by the present study.

V. Concluding remarks

This work presented what is perhaps the most extensive study of the potential surfaces for the hydrolysis of phosphate monoester dianions in solution, providing complete 2 dimensional surfaces for key representative systems. The resulting TSs reproduced the observed LFER in a semi quantitative way, thus lending credibility to the subsequent analysis. The calculated surfaces were found to be quite flat at the TS region, changing between associative and dissociative character depending on the nature of the leaving group. This established that it is unjustified to use the observed LFER to deduce the actual mechanism in a unique way.

Evaluation of the transition state charges establish that the calculated charges and their correlation with the nature of the TS are quite different than what has been assumed in the early insightful studies of physical organic chemists[75, 76] (see below). It is also demonstrated that isotope effects cannot be used in a unique mechanistic analysis of the hydrolysis of phosphate monoester dianions.

Apparently, although early experimental studies were extremely insightful, the resulting insight cannot be used for a detailed analysis and must be augmented by ab initio QM/MM calculations. This problem becomes even more serious when one tries to evaluate the effect of enzyme active sites on the reaction surface for phosphate hydrolysis and /or the effect of different mutations.

This work explored the uniqueness of the effective charge extracted from LFER analysis[75-77]. As mentioned in section IV-b the effective charges provide a useful way for describing the change in charge of the leaving group in cases which can be represented uniquely by two intersecting parabolas. However, in more complex cases they can only be considered as a book keeping description of the observed LFER. This point has been established by evaluating the change in the ab initio charge of the leaving group for associative and dissociative mechanisms. It was found that the effective charges are very similar in both cases. Further studies should involve an EVB analysis of the proper effective charges.

After validating our approach by studies of the solution reaction and in particular the reaction in the presence of the Mg^{2+} ion, we evaluated the surface for the GTPase reaction in the Ras/GAP system. Here it is found that the surface is associative in nature and that again it is impossible to establish by simple experiments (e.g. isotope effect or LFER) whether it is associative or dissociative. Nevertheless, it would be useful to try to reproduce the observed LFER in Ras and to attempt to reproduce the effect of different mutations on the vibrational frequencies of the bound GTP in the associative and dissociative TSs.

The nature of the charge distribution in the TSs of the GTP hydrolysis reaction has been an issue of significant interest as much as the catalytic reaction of Ras is concerned. EVB studies (e.g. refs 9, 88)

that reproduced the experimental catalytic effect found that there is a significant shift of the positive electrostatic potential of the protein towards the leaving group upon transfer of the substrate to the protein active site in both the associative and dissociative paths[9]. In fact, the idea and more importantly the demonstration of a shift of the electrostatic potential was introduced first in ref 89, where it was correlated with the change of the protein between its GDP-bound to GTP-bound structures. At any rate, the shift of the electrostatic potential should lead to a charge shift, and indeed the existence of such a shift has been supported, at least in the reactant state, by careful FTIR studies[19] and quantified in a theoretical work[20]. The present work has not attempted to further quantify the reactant state results of ref. 20 (this is left to subsequent studies) but focused on the implication that the shift of the electrostatic potential and the corresponding catalytic effect indicates that we have a dissociative TS (e.g. ref 78). First, as it seems to be clear from Table 3, the change in charge shift upon moving from the RS to the TS in the RasGAP system is virtually equal for the associative and the dissociative path. Second, true assessment of the catalytic effect in both paths cannot be accomplished without actual calculations of the corresponding energetics for both paths. To the best of our knowledge the only study that addressed this issue has been reported in ref. 9. This study found a similar electrostatic catalysis for the associative and dissociative mechanisms.

Recent studies have examined the energetics of the GTP hydrolysis reaction in solution[32] and in the RasGAP system[17] by a QM/MM approach which involved energy minimization and TS search. These studies were very instructive in pointing out towards an associative TS where the proton is still attacked to the nucleophilic water molecule. However, these studies have not provided the overall free energy surface and underestimated significantly the barrier for the solution reaction. Furthermore, the rate determining steps was found to be very different for the solution and the protein reactions (these rate limiting steps involved a formation of an associative TS in the solution reaction and a proton transfer to the leaving group in the protein reaction). At present it is hard to pinpoint the source of the difference between the results of these studies and the results of the present study. We can only point out that in view of the complexity of the system studied, we placed so much emphasis on calibrating and validating our calculations using the solution LFER and examining the results using several approaches. No such validation effort was reported by other studies.

As should be clear from the above discussion we believe that the present study can be considered as the most systematic available computational analysis of the hydrolysis of phosphate monesters dianions in water. The reason for our view is not only the fact that we reproduce nicely the observed rate constants for very different systems, but also our estimate of the error range (section IVd) by using quite different approaches. We also like to reemphasis that our study is not base on any gas phase transition state search but on a very careful mapping of the surface of the solvated system. Having a full surface rather than just isolated TSs is in our view a major advantage of the present treatment. It is also important to mote that that we do not base our claim for reasonable accuracy on the accuracy of the DFT calculations which is clearly not perfect but on the compounded experience from modeling the overall experimental trend. This point is particularly important when we address the effect of the protein on the calculated trend, In this respect it is useful to mention that we were able to reproduce the every large solvent screening of the repulsion between the negative charges of the reacting system since we reproduce the experimental barrier in the highly charged mono methyl pyrophosphate trianion system.

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There has been a significant interest in the validity of the substrate as a base proposal (e.g. refs 14, 90) for Ras and related systems. In some studies (e.g. ref 40) it was argued that there are experimental evidences against such a mechanism in solution. However, it has been clarified (e.g. ref 9) that there is no direct experiment that can establish this issue. There is, nevertheless, a point of clarification. That is, the substrate as a base idea addresses the fact that the proton transfer (PT) step in Ras does not involve Gln61 as a base[12] and that the proton is transferred in one way or another to the γ-phosphate. As discussed in refs 41 and 9, this transfer can be done in a concerted way with the nucleophilic attack and

still be considered as a phosphate as a base mechanism (as long as the mechanism is an associative mechanism). The phosphate as a base mechanism does not require any preequilibrium PT step as implied in some works (e.g. ref 16) and only requires that the PT will occur on the way or near the associative TS. It is also important to clarify that, in contrast to early assumptions (see discussion in ref. 91), it is impossible to determine experimentally whether the water attack occurs in a stepwise mechanism or a concerted way (since it is impossible to examine the OH^- attack on a protonated phosphate due to conflicting pH requirements). At any rate, as already clarified in ref 9 the main issue is whether we have an associative or dissociative TS in the Ras and RasGAP reactions, and whether Gln61 participates in the reaction in a direct way.

The dissociative mechanism has been frequently associated with a transition state with a protonated TS[78]. It has also been suggested that this is a well established experimental fact for the solution reaction (e.g. refs 78, 87) and that it is consistent with the observed isotope effect[31]. While in the case of a leaving group with a very large pK_a we may have a fully dissociative mechanism and the proton of an attacking water may be transferred to the negatively charged leaving group, the situation is quite different in cases of S_N2 and/or associative reactions, where the first TS involves a concerted PT to the phosphate or a transition state where the proton is not yet transferred.

Obviously the analysis presented here is not the last word in the exploration of the nature of the TS in the hydrolysis of phosphomonoesters in solutions and proteins. Further QM/MM calculations should explore the effect of the solute entropy by FEP approaches that do not fix the solute[60]. However, we believe that the present systematic study has helped to advance our understanding of the nature of the reaction path and that the analysis of the type presented here is crucial for further progress in studies of G-proteins and related systems.

It is also useful to note that the present study provided compelling reminders about the major difference between experimental information and the interpretation of such experiments. As shown here in several key examples (e.g. the analysis of LFERs and isotope effects) it is not justified to accept traditional interpretations of experimental result as experimental facts. In the case of high energy TSs it is crucial to add computational studies as a key tool in the interpretation of experimental results.

Acknowledgment

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Supporting Information Available

Complete Ref. 71, Figures of TS structures, Tables of PES surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

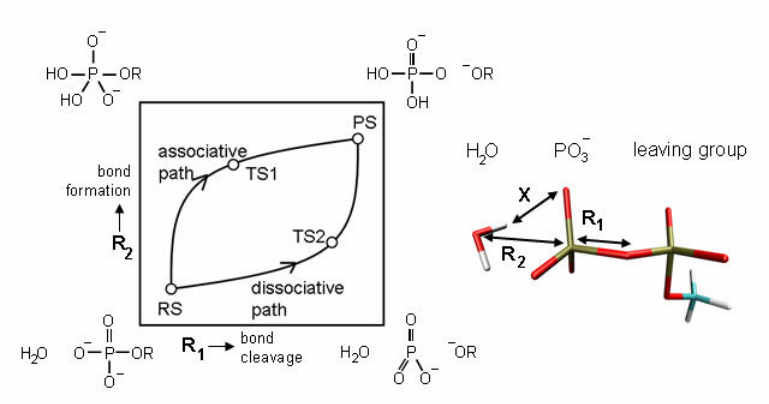


Figure 1. A schematic description of the potential surface for the hydrolysis of phosphomonoesters. The figure provides a clear definition of the associative and dissociative paths and also defines the three reaction coordinates R_1 and R_2 and X.

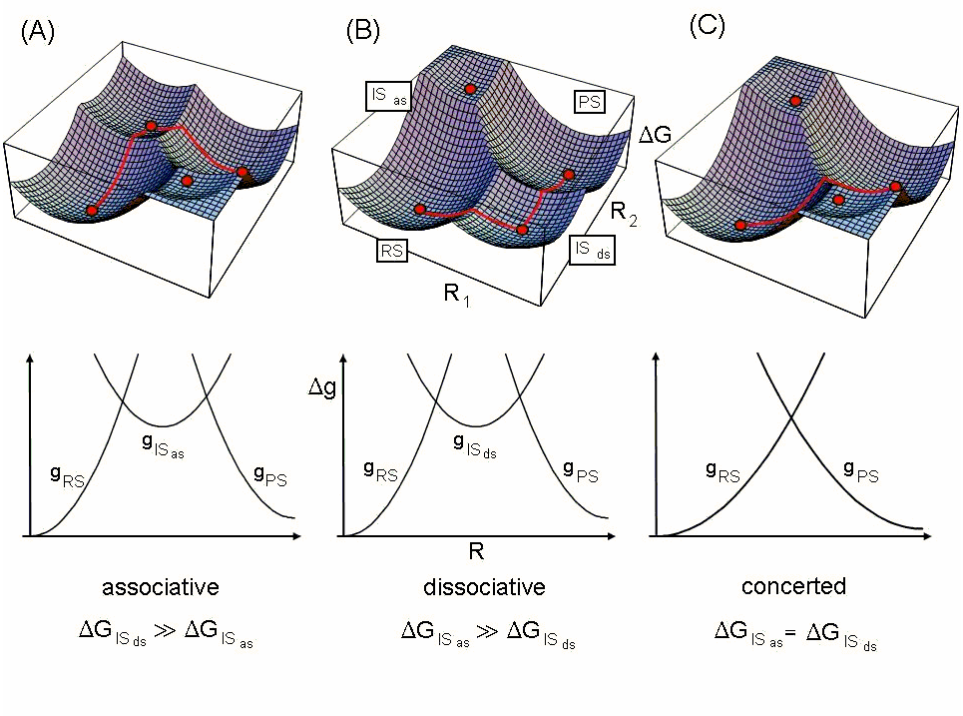


Figure 2. A VB description of the energy surfaces for phosphate hydrolysis in three extreme cases, associative (A), dissociative (B) and concerted (C). The figure generates the surfaces for the hydrolysis reaction by mixing four states using an EVB formulation (the effect of the off-diagonal term is in included for simplicity) . When the surface follows one of the extreme cases it is relatively easy to describe the corresponding LFER by shifting parabolas in the lower part of the surface as was done in ref. 8. RS, IS and PS represent, respectively, reactant, intermediate and product states. The indices as and ds stand for associative and dissociative, respectively. The dots on the surfaces designate the minima of the corresponding diabatic surfaces

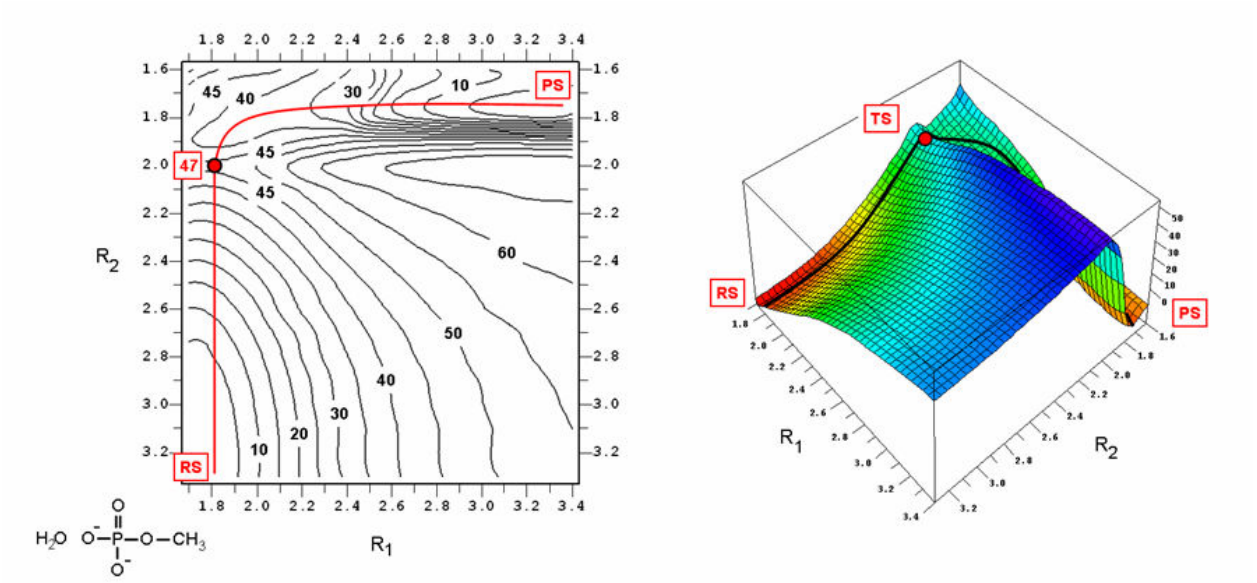


Figure 3. The calculated surface for the hydrolysis of the mono methyl phosphate dianion in the space defined by R_1 and R_2. The figure depicts the reaction path that connects the RS with the PS and the corresponding TS. Energies are given in kcal/mol and distances in Å.

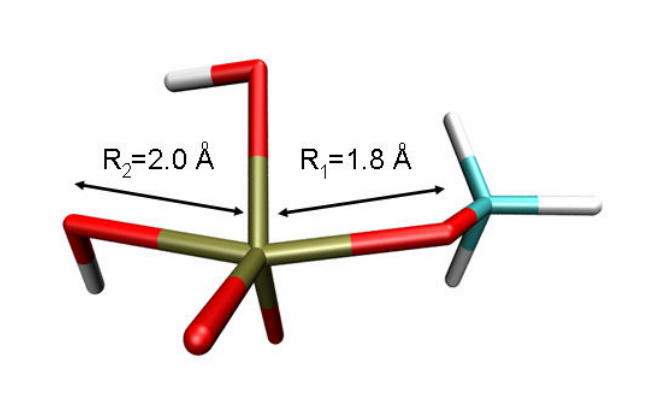


Figure 4. The calculated TS structure for the hydrolysis of the mono methyl phosphate dianion.

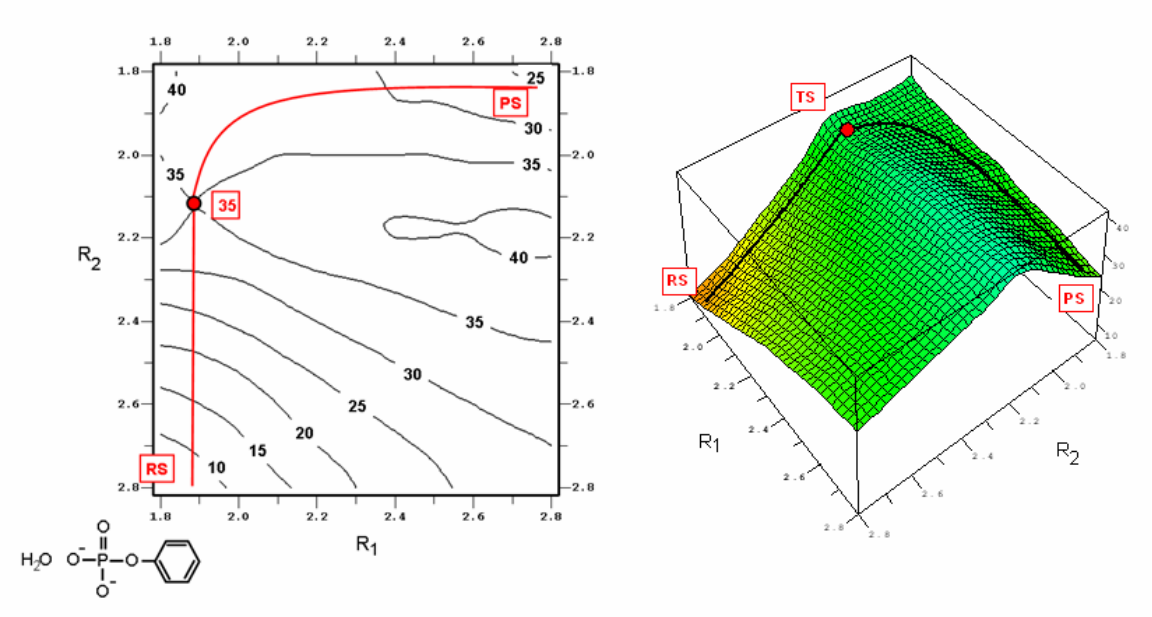


Figure 5. The calculated surface for the hydrolysis of the phenyl phosphate dianion in the space defined by R_1 and R_2. The figure shows the reaction path that connects the RS with the PS and the corresponding TS. Energies are given in kcal/mol and distances in Å.

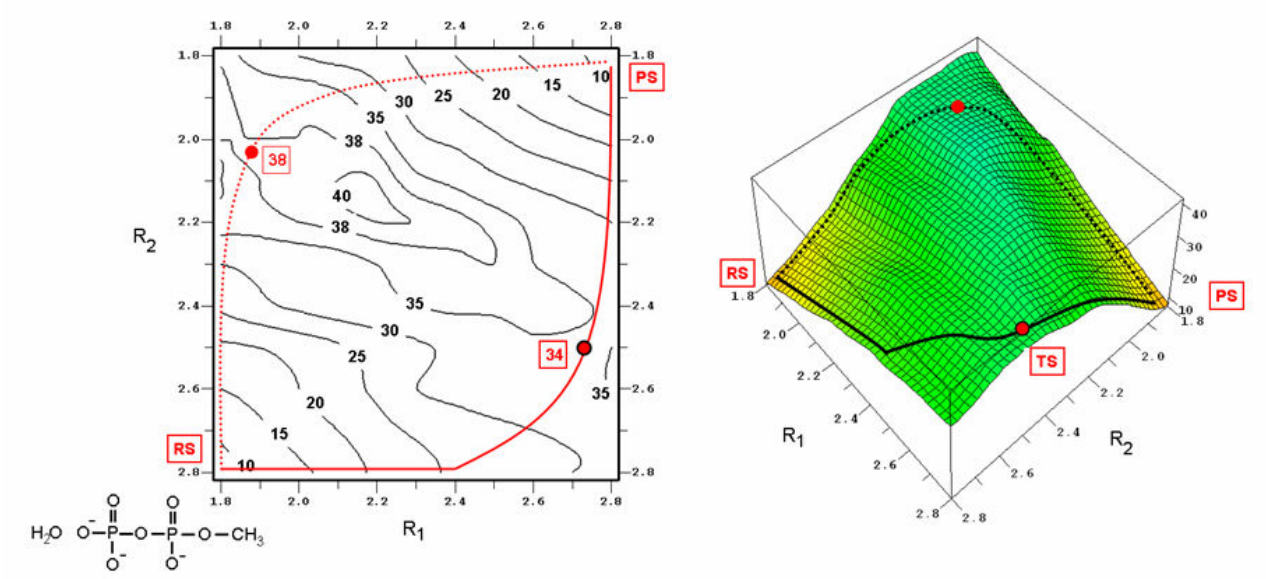


Figure 6. The calculated surface for the hydrolysis of the mono methyl pyrophosphate trianion in the space defined by R_1 and R_2. The figure shows the reaction path that connects the RS with the PS and the corresponding TS. An alternative associative path with higher energy barrier is shown (dashed line). Energies are given in kcal/mol and distances in Å.

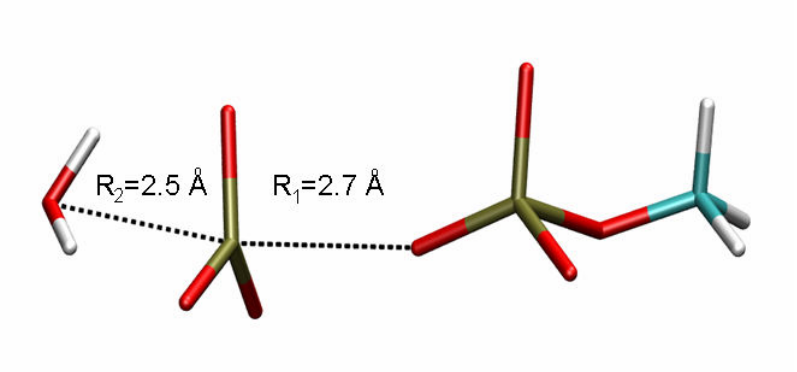


Figure 7. The calculated TS structure for the hydrolysis of the mono methyl pyrophosphate trianion.

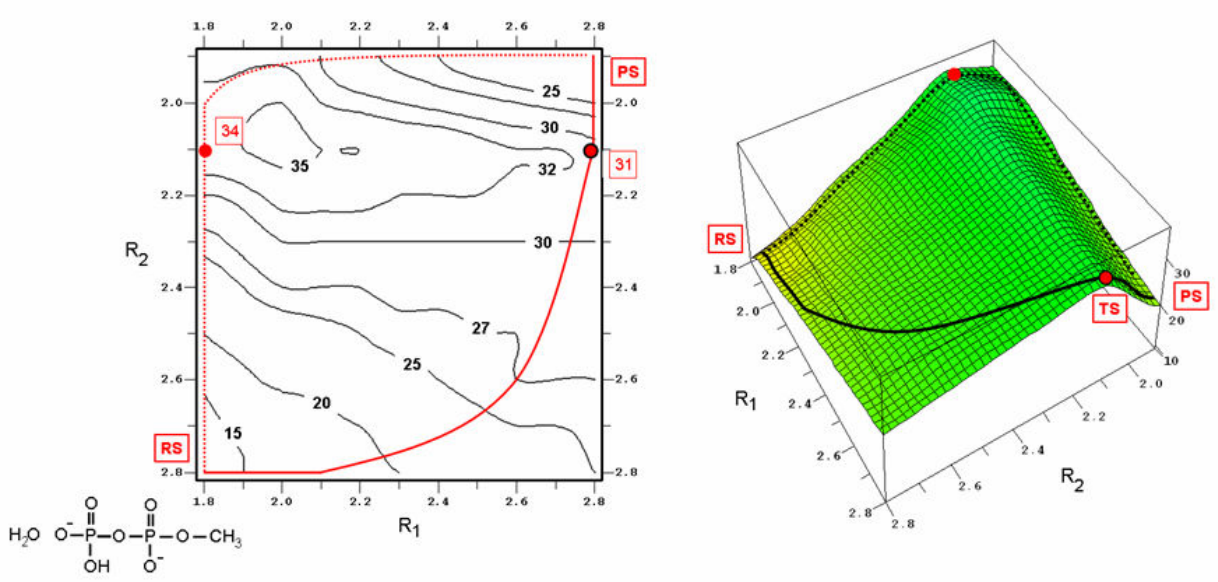


Figure 8. The calculated surface for the hydrolysis of the mono methyl pyrophosphate dianion in the space defined by R_1 and R_2. The figure shows the reaction path that connects the RS with the PS and the corresponding TS. An alternative associative path with higher energy barrier is shown (dashed line). Energies are given in kcal/mol and distances in Å.

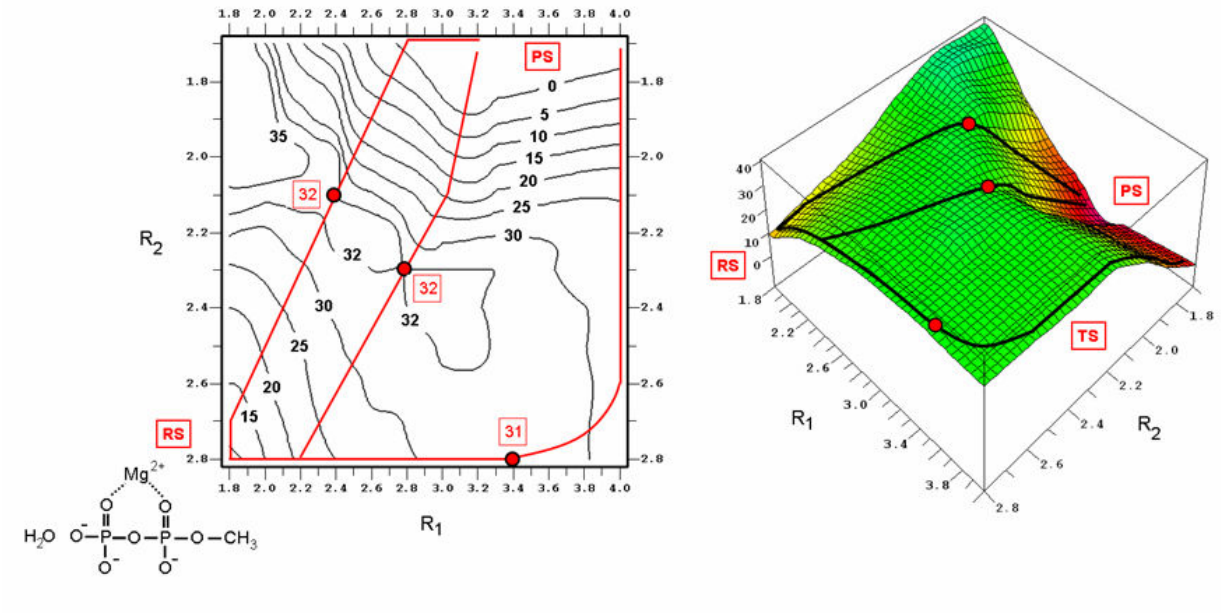


Figure 9. The calculated surface for the hydrolysis of the magnesium mono methyl pyrophosphate trianion in the space defined by R_1 and R_2. The figure shows three possible reaction paths with similar energy barriers that connect the RS with the PS and the corresponding TSs. Energies are given in kcal/mol and distances in Å.

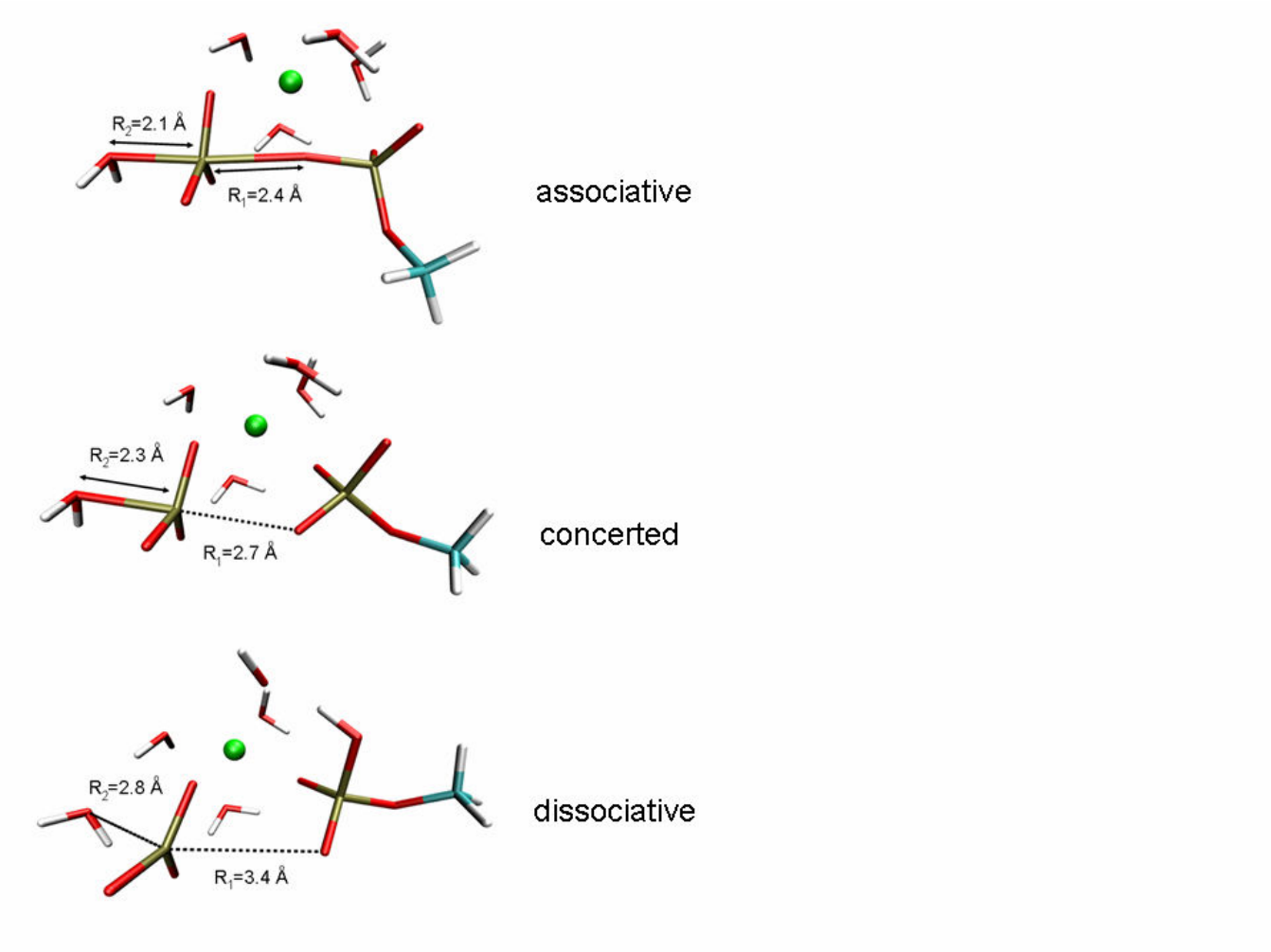


Figure 10. Three possible calculated TSs structures for the hydrolysis of the magnesium mono methyl pyrophosphate trianion.

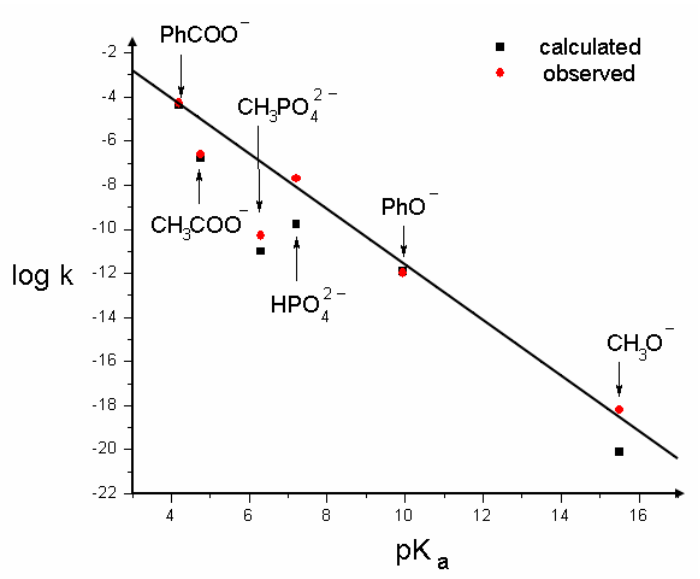


Figure 11. Comparison of calculated (square boxes) with observed (filled circles) LFER (i.e. the correlation between the log k of the different reactions and the corresponding pK_a s of the leaving groups) for phosphate monoester dianions with different leaving groups. The rate constants are given at 39 C° in units of s^{-1}. The corresponding data is given in Table 2. The linear curve is taken from ref. 46 where it was obtained as a least-square fit (log k=1.0-1.26 pK_a) to measured hydrolysis rate constants of several phosphomonoesters.

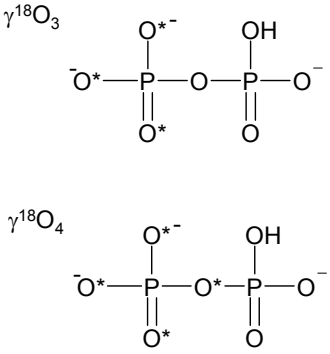


Figure 12. Defining the isotopic labeling of the pyrophosphate oxygens used in Table 4 and Table 5. The oxygen atoms marked with an asterisk are substituted with an ^{18}O isotope whereas all other oxygen atoms are left with ^{16}O.

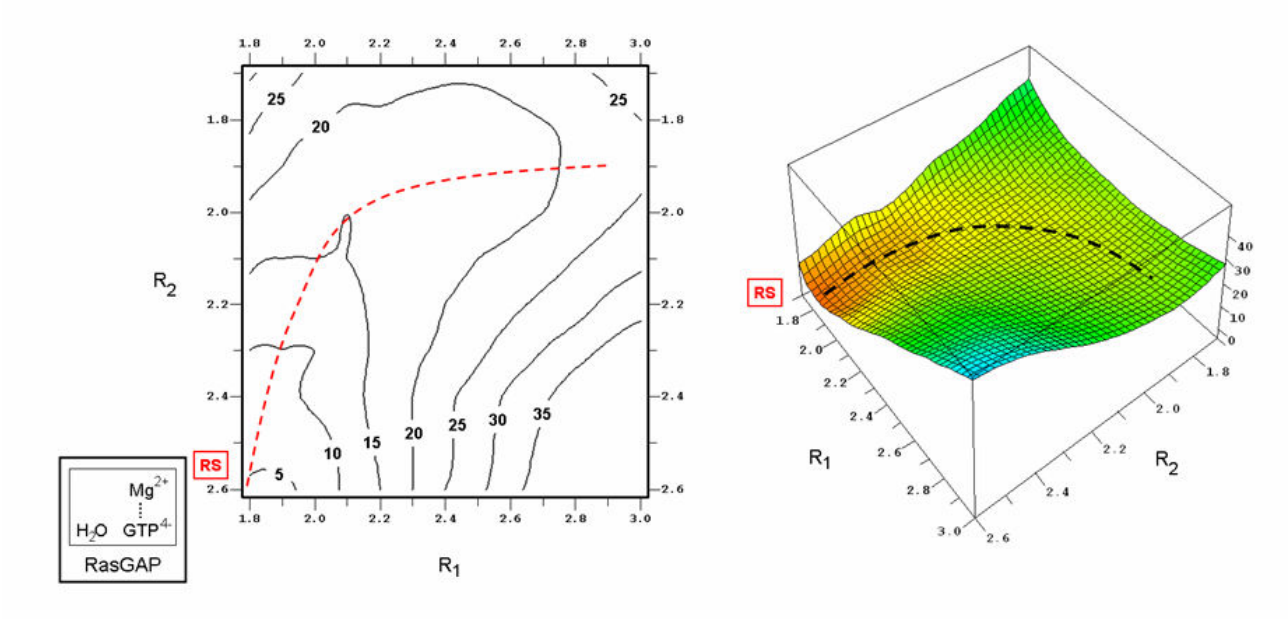


Figure 13. The calculated surface for the hydrolysis of GTP in RasGAP in the space defined by R_1 and R_2. The figure shows a part of the reaction path (dashed line). The PS region is shown in Fig. 14. Energies are given in kcal/mol and distances in Å.

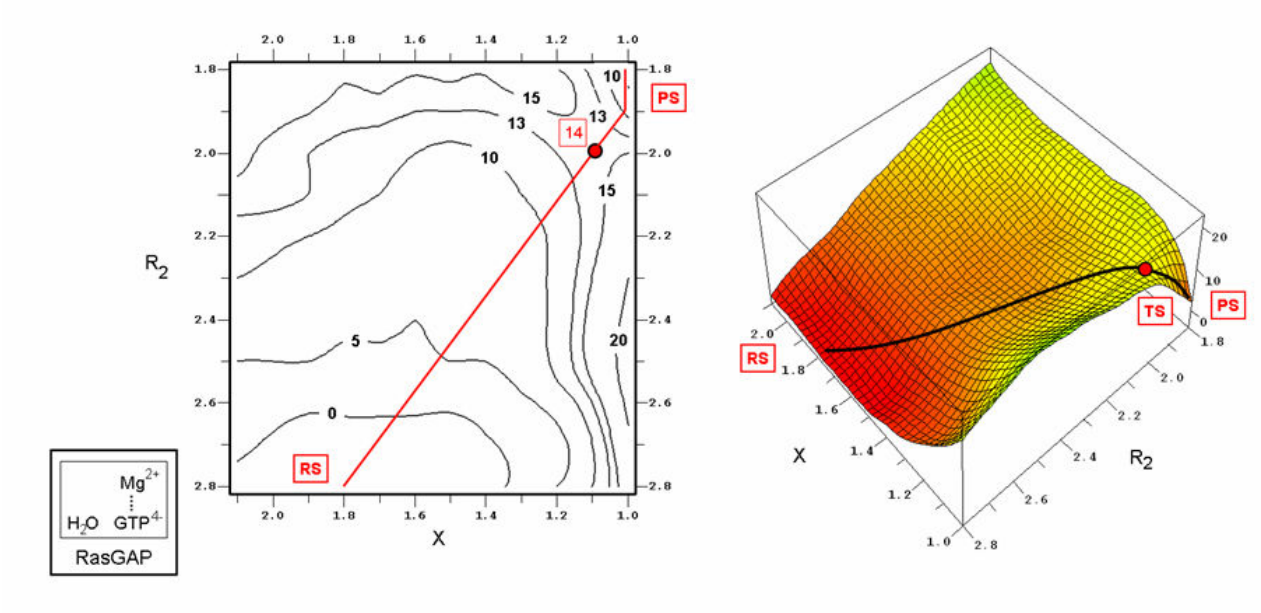


Figure 14. The calculated surface for the hydrolysis of GTP in RasGAP in the space defined by the proton transfer coordinate, X and R_2, fixing R_1 at the TS distance at about 2.1 Å. The figure shows the reaction path that connects the RS with the PS and the corresponding TS. Energies are given in kcal/mol and distances in Å. (See method section for more details about our mapping procedure.)

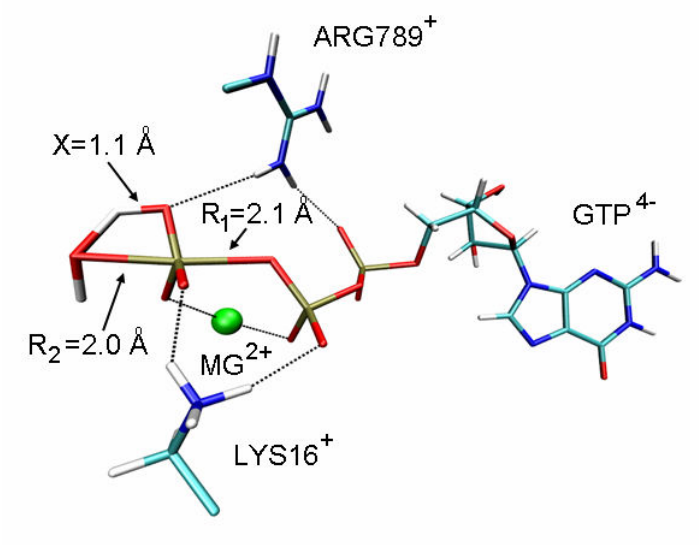


Figure 15. The calculated TS structure for the hydrolysis of the GTP substrate in RasGAP.

Table 1. Comparing the energies obtained by different solvent models and different ab initio methods for characteristic points on the PES of the hydrolysis of the magnesium mono methyl pyrophosphate trianion.[a]

	COSMO			LD	FEP
structure	**B3LYP/ 6-311++G****	MP2/ 6-311++G**	B3LYP/ Lan2DZ		
RS	0	0	0	0	0
associative TS	32	36	33	33	35
concerted TS	31	36	35	32	34
dissociative TS	31	33	36	31	35
completely dissoc. TS	31	33	34	31	34
PS	2	-3	3	-1	7

[a] The energies are given in kcal/mol. The different solvent models (COSMO, LD and FEP) are discussed in the method section. The B3LYP/6-311++G** has been the method of for the evaluation of all the PESs.

Table 2. The correlation between the rate constants (and activation barriers) and the observed pK_a values for the hydrolysis reaction of phosphomonoesters with different leaving groups.[a]

Leaving group	pK_a	$\Delta G^{\neq}_{calc}$	$\Delta G^{\neq}_{exp}$	log k_{calc}	log k_{exp}
CH_3O^-	15.5 [g]	47	44 [b]	-20.1	-18.2 [b]
PhO^-	10.0 [g]	35	35 [b]	-11.9	-12.0 [b]
$CH_3PO_4^{2-}$	6.3 [g]	34	33 [c]	-11.0	-10.3 [c]
HPO_4^{2-}	7.2 [g]	32	29 [f]	-9.8	-7.7 [f]
$(Mg^{2+})CH_3PO_4^{2-}$ [i]	6.5 [h]	31	28 [e]	-8.9	-6.8 [e]
CH_3COO^-	4.8 [g]	28	28 [d]	-6.8	-6.6 [d]
$PhCOO^-$	4.2 [g]	25	24 [d]	-4.4	-4.3 [d]

[a] The rate constants are given in s^{-1} and calculated for 39C°. The energies are given in kcal/mol and the observed values are obtained from the observed rate constants using transition state theory. The reacting system includes an attacking water molecule, a phosphate dianion group and the indicated leaving group. The sources of the observed rate constants are refs. 46, 39, 92, 74and 48 for b, c, d, e and f, respectively. The observed pK_a values are taken from ref. 93and 94 for g and h.

[i] This system involves a Mg^{2+} ion and is compared to measurements of solvated GTP[74].

Table 3. The charges of the leaving group in different states of the hydrolysis of some selected systems.[a]

	mono methyl phosphate dianion	mono methyl pyrophosphate trianion	mono methyl pyrophosphate trianion[b]	GTP in solution tetra-anion	GTP in RasGAP tetra-anion
	leaving groups				
state	CH_3O	PO_4CH_3	PO_4CH_3	$PO_4PO_3CH_3$	$PO_4PO_3CH_3$
RS	-0.29	-1.44	-1.42	-2.48	-2.51
	(-1.71)	(-1.56)	(-1.58)	(-1.52)	(-1.49)
associative TS	-0.51	-1.74	-1.86	-2.73	-2.79
	(-1.49)	(-1.26)	(-1.14)	(-1.27)	(-1.21)
dissociative TS	-0.97	-1.89	-1.72	-2.87	-2.83
	(-1.03)	(-1.11)	(-1.28)	(-1.13)	(-1.17)
PS		-1.88	-1.69	-2.88	-2.89
		(-1.12)	(-1.31)	(-1.12)	(-1.11)

[a] The charges are given in the unit of the elementary charge. The reported charges of the leaving group are taken as the sum of the partial charges of all the atoms of the leaving group, for different points on the PES for the hydrolysis reaction. The charge of the remaining part of the system (i.e. the phosphate and hydrolyzing water molecule) is given in parenthesis. The charges are evaluated according to the Merz-Singh-Kollman scheme.

[b] This system contained a Mg^{2+} ion with its hydration shell (i.e. four additional water molecules). The charge transferred to the magnesium ion and its hydration shell was added in equal halves to the leaving group and the remaining system consisting of the phosphate and the hydrolyzing water molecule.

Table 4. Difference of zero point energies (relative to the reactant state) for different TSs of the magnesium mono methyl pyrophosphate trianion.[a]

isotopic composition	associative TS		dissociative TS	
	GA	SA	GD	SD
$\gamma^{16}O_4$	-0.130	-0.609	-1.037	-1.973
$\gamma^{18}O_3$	-0.136	-0.625	-1.040	-1.978
$\gamma^{18}O_4$	-0.137	-0.628	-1.039	-1.980

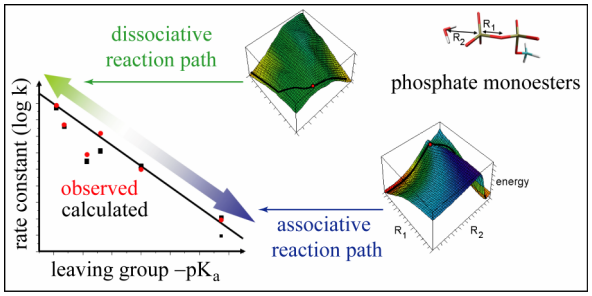
[a]The zero point energies are given in kcal/mol. $\gamma^{16}O_4$ denotes the phosphate with the natural ^{16}O isotope, $\gamma^{18}O_3$ and $\gamma^{18}O_4$ are the ^{18}O labeled atoms according to the notation of Fig. 12. The calculations considered structures that differ from each other only slightly for both, the associative and the dissociative mechanism (this allows us to examine the sensitivity of our calculated values to small structural changes). GA and GD are gas phase optimized and subsequently solvated TSs, while SA and SD are solution optimized TSs (here G and S designates gas and solution respectively) . The corresponding structures are depicted in the supplementary material.

Table 5. Calculated isotope effects for the systems considered in Table 4.[a]

isotopic composition	associative structures		dissociative structures	
	GA	SA	GD	SD
$\gamma^{18}O_3$	1.0092	1.0256	1.0061	1.0081
$\gamma^{18}O_4$	1.0122	1.0308	1.0041	1.0112

[a] $\gamma^{18}O_3$ and $\gamma^{18}O_4$ are the ^{18}O labeled ions according to the scheme of Fig. 12. The different structures are defined in the caption of Table 4.

References



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