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A Theoretical Comparison of *p*-Nitrophenyl Phosphate and Sulfate Hydrolysis in Aqueous Solution: Implications for Enzyme Catalyzed Sulfuryl Transfer

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ABSTRACT

Both phosphoryl and sulfuryl transfer are ubiquitous in biology, being involved in a wide range of processes, ranging from cell division to apoptosis. Additionally, it is becoming increasingly clear that enzymes that can catalyze phosphoryl transfer can often cross-catalyze sulfuryl-transfer (and *vice versa*). However, while there have been extensive experimental and theoretical studies performed on phosphoryl transfer, the body of available research on sulfuryl transfer is comparatively much smaller. The present work presents a direct theoretical comparison of *p*-nitrophenyl phosphate and sulfate monoester hydrolysis, both of which are considered prototype systems for probing phosphoryl and sulfuryl transfer respectively. Specifically, free energy surfaces have been generated using density functional theory, by initial geometry optimization in PCM using the 6-31+G* basis set and the B3LYP density functional, followed by single point calculations using the larger 6-311+G** basis set and the COSMO continuum model. The resulting surfaces have been then used to identify the relevant transition states, either by further unconstrained geometry optimization, or from the surface itself where possible. Additionally, configurational entropies were evaluated using a combination of the quasiharmonic approximation and the restraint release approach and added to the calculated activation barriers as a correction. Finally, the overall activation entropy was estimated by approximating the solvent contribution to the total activation entropy using the Langevin dipoles solvation model. We have reproduced both the experimentally observed activation barriers and the observed trend in the activation entropies with reasonable accuracy, as well providing a comparison of calculated and observed ¹⁵N and ¹⁸O kinetic isotope effects. We demonstrate that, counterintuitively, the hydrolysis of the *p*-nitrophenyl *sulfate* proceeds through a more expansive pathway than its phosphate analogue. Additionally, we show that the solvation effects upon moving from the ground state to the transition state are quite different for both reactions, suggesting that the enzymes that catalyze these reactions would need active

sites with quite different electrostatic preorganization for the efficient catalysis of either reaction (despite which many enzymes can catalyze both phosphoryl and sulfuryl transfer). We believe that such a comparative study is an important foundation for understanding the molecular basis for phosphate-sulfate cross promiscuity within members of the alkaline phosphatase superfamily.

Keywords: phosphate hydrolysis • sulfate hydrolysis • DFT calculations • More O’Ferrall-Jencks plots • enzyme promiscuity

I. INTRODUCTION

Phosphate ester hydrolysis is ubiquitous in biology, being involved in protein synthesis, signal and energy transduction, as well as the replication of the genetic material¹⁻⁵. As a result, phosphate esters have been the subject of extensive experimental^{3,6-13} and theoretical¹⁴⁻³² studies (though the precise mechanisms of both the solution and enzyme catalyzed reactions remain controversial, see e.g. ^{6-8,12,14,15,25,26,29,33-35}). In contrast, while there has been some significant work on the hydrolysis of sulfate esters (see e.g. ^{27,33,36-39} and references cited therein, amongst others), comparatively less attention has been paid to this class of reaction, despite the fact that, like phosphate esters, sulfate esters are of critical biological importance, playing for instance roles in detoxification⁴⁰, the biosynthesis of steroids⁴¹ and cellular signaling³⁶.

Phosphate esters are generally rather unreactive under physiological conditions^{8,9,42-45}, making the enzymes that catalyze this class of reaction extremely proficient. As an example, the uncatalyzed hydrolysis of dimethyl phosphate is estimated to have an upper limit of $\sim 1 \times 10^{-15} \text{ s}^{-1}$ for spontaneous P-O cleavage at 25° C⁴⁶, with enzymes such as Staphylococcal Nuclease accelerating the rate of reaction by at least 10^{16} -fold⁴⁷ (in this particular case, for instance, computational studies find a barrier reduction of more than 35 *kcal/mol* compared to the corresponding uncatalyzed reaction in solution^{48,49}, with the most detailed existing analysis of the molecular basis for catalysis by this enzyme being presented in

Ref. ⁴⁹). Clearly, understanding the source of such tremendous catalytic proficiency is a question of major importance, though the main contributor to this proficiency appears to be the existence of a highly preorganized active site⁴⁹. In addition to the high catalytic efficiency of many phosphatases, several also exhibit promiscuous sulfatase activity⁵⁰. Conversely, as was recently demonstrated for instance in the case of arylsulfatase⁵¹, there exist sulfatases that can catalyze phosphoryl transfer. It has been argued that such enzyme promiscuity plays an important role in the evolution of proteins⁵²⁻⁵⁴. Therefore, understanding how these enzymes not only identify their native substrates but also select for and discriminate between their promiscuous substrates holds a key to understanding evolution of function within enzyme superfamilies. Prior to attempting to understand the enzyme catalyzed reactions, however, it is important to have a clear understanding of the corresponding reference reactions in solution^{9,49}, not only in order to obtain a thorough understanding of the basic chemistry of the reaction in the absence of the enzyme and to be able to quantify the catalytic proficiency of the enzyme, but also, where relevant, for the calibration and validation of the theoretical approaches that are being used with which to probe the corresponding enzyme catalyzed reaction. Experimental data on aryl sulfates^{37-39,55}, particularly linear free energy relationships (LFER), would suggest that the mechanism of hydrolysis of sulfate monoester anions is similar to that of phosphate monoester dianions^{33,56}. However, this does not rationalize the large difference in activation entropy between the two^{33,56} (-18.5 eu for the pH-independent hydrolysis of the *p*-nitrophenyl sulfate (pNPS) anion compared to +3.5 eu for the hydrolysis of the *p*-nitrophenyl phosphate dianion (pNPP), where “eu” refers to entropy units, with 1 eu corresponding to 1 cal K⁻¹ mol⁻¹), which, based on the traditional interpretation, would suggest greater nucleophile involvement in the case of the hydrolysis of the sulfate anion than in the case of its phosphate counterpart. It should be noted that previous studies have demonstrated that the interpretation of LFER data (particularly in the case of concerted mechanisms) should be approached with care^{14,16-18,21} and also that both associative and dissociative pathways can have similar activation entropies⁵⁷. This work presents a direct theoretical comparison of the hydrolysis of the *p*-nitrophenyl phosphate and

sulfate monoesters, which form reference reactions for the native/promiscuous activities of a number of members of the alkaline phosphatase superfamily. From the examination of the relevant transition states, it appears that, surprisingly, the hydrolysis of the sulfate monoanion proceeds through a more expansive pathway than the phosphate dianion. However, we have reproduced both the experimental activation energies and activation entropies with reasonable accuracy. This further emphasizes the trend observed in our previous studies, which suggest that (at least in the case of phosphoryl transfer) the qualitative interpretation of experimental data can be ambiguous^{14-18,21,24,57,58}, highlighting the need for coupling experimental work with detailed theoretical studies. It is believed that such a comparative study of phosphate and sulfate hydrolysis is an important first step towards trying to understand the factors that govern enzymatic phosphate-sulfate promiscuity, which in itself is a potentially important step towards trying to understand the evolution of function within enzyme superfamilies.

II. THEORETICAL BACKGROUND

II.1 MECHANISTIC CONSIDERATIONS

Despite the fact that phosphoryl transfer appears to involve a simple nucleophilic displacement, studies on this class of reaction are complicated by the fact that the availability of low-lying d-orbitals on the phosphorus atom allows for the existence of pentavalent phosphorous species as intermediates in the hydrolysis mechanism. Thus, in the two limiting cases, phosphoryl transfer can proceed *via* either stepwise associative (A_N+D_N) or dissociative (D_N+A_N) mechanisms, proceeding through penta- or trivalent intermediates respectively. Additionally, the reaction can proceed through a concerted (A_ND_N) pathway in which the bond-cleavage and bond-formation processes occur in a single transition state, without the involvement of an intermediate. Such pathways can in turn also be associative or dissociative in nature, depending on whether the reaction is dominated by bond-cleavage to the leaving

group or bond-formation to the nucleophile as the system moves from the ground state to the transition state. However, classifying such transition states as associative or dissociative is not always clear cut and can be challenging, and thus, for simplicity, throughout this work, when referring to concerted pathways, instead of “associative” and “dissociative”, the terms “compact” and “expansive” will be used, with the term “dissociative” being reserved specifically for D_N+A_N pathways proceeding through a true metaphosphate intermediate, following the nomenclature of, for instance, Westheimer⁶¹. It should be emphasized, however, that the use of this terminology is not intended to in any way imply a cut-off between two mechanistic possibilities (as is the case with the stepwise pathways), but rather simply to highlight the fact that concerted pathways lie on a spectrum between more compact (associative) and more expansive (dissociative) pathways.

Unsurprisingly, in light of their biological importance, the hydrolysis of phosphate esters has been subjected to extensive investigation (see e.g. Refs. ^{2,34,62,63}, and references cited therein). Experimental studies have revolved around a range of established techniques for elucidating reaction mechanisms, including the use of linear free energy relationships (LFER)⁶⁴, kinetic isotope effects^{2,65}, and examining entropies of activation⁷. However, the extent to which such tools can (even in combination) provide clear mechanistic insight has been questioned¹⁴, and in fact computational studies show that the interpretation of such mechanistic markers is far from clear, in part due to the fact that in many cases, phosphoryl transfer reactions can potentially occur through multiple equally viable mechanisms, even in solution^{24,57}, and, additionally, in the case of concerted pathways, LFER can mask gradual changes in the nature of the transition state¹⁶⁻¹⁸. Therefore, while clearly important, such markers need not have unique mechanistic interpretations. This problem can be partially mitigated by the use of More O’Ferrall Jencks (MFJ) plots^{66,67}, which map the reaction surface in the space defined by any two distinguished reaction coordinates and allows for the direct comparison of any viable mechanisms, if more than one is available to the system. The present work maps out such surfaces for the hydrolysis of both the *p*-nitrophenyl phosphate and the *p*-nitrophenyl sulfate anions as a function of phosphorus/sulfur – oxygen

distances to the incoming nucleophile and departing leaving group. Computational studies that examine such surfaces for the hydrolysis of phosphate monoester dianions have suggested that the reaction proceeds through concerted A_ND_N pathways that can be compact or expansive in nature depending on the acidity of the leaving group^{16,57}. Additionally, it has been suggested based on experimental data that the mechanism of hydrolysis of sulfate monoester anions is similar to that of their phosphate monoester dianion analogues (for discussion, see^{33,68} and references cited therein). Therefore, it could be assumed that, in both cases, the reaction would proceed through more expansive concerted pathways. The advantage of generating an MFJ plot over examining isolated transition states and intermediates is that no pre-assumption is made with regard to the reaction mechanism, and, additionally, when the system can be successfully described in terms of two reaction coordinates, it is possible to identify all viable mechanisms (when there is more than one) using the same nucleophile and substrate, on the same surface. This is particularly important as outlined above in the complex case of phosphoryl transfer reactions, and such an approach has already been demonstrated to be highly successful in studies of phosphate hydrolysis. Herein, we extend our study to also encompass sulfuryl transfer, as this also allows for a direct comparison of the respective surfaces at the same level of theory.

II.2 COMPUTATIONAL DETAILS

2-dimensional surfaces for water attack on pNPP and pNPS were generated in the space defined by the phosphorus/sulfur – oxygen distances to the departing leaving group (x-axis) and incoming nucleophile (y-axis) respectively. At each point on the surface, these two distances were constrained and all other degrees of freedom were allowed to freely optimize. Both free-energy surfaces were generated by careful reaction coordinate pushing, mapping from 1.6 to 3.0Å, using a 0.15Å distance increment, with relative energies and geometries being examined at each point on the plot in order to minimize noise generated by variation in unconstrained degrees of freedom, and to verify that the true minimum energy surface is

obtained. All energies are given relative to the sum energies of the nucleophilic water molecule and either pNPP or pNPS as relevant, at infinite separation. Note that in both cases generating the correct surface is complicated by the presence of a potential proton-transfer from the incoming nucleophile onto the substrate, and if one were to be extremely careful about this, one would consider a 3-dimensional reaction cube (as suggested by for instance Guthrie⁶⁹), where the proton position would be implicitly included as part of the definition of the reaction coordinate. However, the computational cost of such a setup would be prohibitive due to the extremely large number of individual calculations required. Additionally, setting this system up as a cube would essentially be forcing a proton transfer that might not necessarily be desirable. Finally, even if an intramolecular general base mechanism is defined as a distinguishable path, this is not an independent pathway, as the substrate is still the ultimate proton acceptor, and this property will change as the reaction progresses. To avoid this, therefore, the proton transfer was not implicitly included in the definition of the reaction coordinate, as was also done in previous works where proton transfer has been an issue (e.g. Refs. ^{16,24,57}).

All *ab initio* calculations were performed using Gaussian03⁷⁰, and Becke's three-level (B3LYP) hybrid functionals⁷¹. These utilize a combination of Hartree-Fock exchange and density functional theory (DFT) exchange correlation. Initial geometries were obtained using the 6-31+G* basis set and the polarized continuum model⁷²⁻⁷⁵ (PCM) with integral equation formalism. This allows for the calculation of the analytical gradients of the solute-solvent surface. The inclusion of a continuum model in the initial geometry optimizations was particularly necessary in the case of the highly charged phosphate monoester dianion, which tended to dissociate under gas-phase optimization (it is believed that this is a simulation artifact). The energetics of the resulting structures were then evaluated using single point calculations with the larger 6-311+G** basis set and the COSMO continuum model^{76,77}. The choice of COSMO rather than PCM for the corrected energies was due to the fact that our validation study in the case of phosphate diesters¹⁷ shows that this model is able to effectively screen charge-charge interactions and provide reasonable solvation free energies. Additionally, this approach has been

successful in our previous studies of phosphoryl transfer^{17,20,21,24,57}. Finally, in all cases, the UFF model was used rather than the standard UA0 model. This model, which is based on radii from the UFF force field⁷⁸, places spheres on all hydrogen atoms, thus treating them explicitly, and allows for potential proton transfer to be accounted for. As an aside, it should be noted that it might be tempting to include explicit water molecules in the calculation to reproduce the first solvation shell, as was done in e.g. Ref. ⁷⁹ (amongst others). However, as was demonstrated in Ref. ²⁰, calculations involving mixed implicit/explicit solvent are problematic, with the inclusion of extra water molecules being likely to adversely affect the accuracy of our results. Therefore, the choice of using a “pure” implicit solvation model was a conscious one, and is in line with our previous work.

At this point, it should be noted that when studying these systems, it is usually important to take the activation entropy of the reaction into account, as the contribution this makes is often non-negligible. This is particularly relevant in the present case, due to the large discrepancy between the activation entropies in the case of the two substrates^{33,56}. However, evaluating the activation entropy in a reliable and accurate way presents a significant computational challenge. Specifically, the activation entropy is composed of two components. The first of these is the solvation entropy, $T\Delta S_{solv}^{\ddagger}$, which measures the *solvent* contribution to the overall activation entropy. The continuum solvation model already implicitly accounts for this contribution, however, in order to be able to approximately quantify this contribution, we have also used the procedure of Ref. ⁵⁷ and evaluated $T\Delta S_{solv}^{\ddagger}$ using the Langevin dipole solvation model⁸⁰ (the accuracy of which has been evaluated using extensive benchmarks⁸⁰), comparing the transition state to the reactant and nucleophile at infinite separation. The second component is the configurational entropy, $T\Delta S_{conf}^{\ddagger}$, which measures the *solute* contribution to the activation entropy. In this work, we have followed the strategy used in our previous studies of phosphoryl transfer (e.g. Refs. ^{18,57} amongst others), and evaluated $T\Delta S_{conf}^{\ddagger}$ using a combination of the quasiharmonic (QH) and restraint release (RR) approaches^{81,82}, which are obtained from molecular dynamics simulations using

explicit solvent. This approach, which has been described in detail elsewhere (see e.g. ^{81,82}), imposes strong harmonic Cartesian restraints on both the reactant (RS) and transition (TS) state structures, and subsequently evaluates the free energy of releasing these restraints using a free energy perturbation (FEP) approach. The resulting configurational entropy can then be obtained using Eq. 1:

$$-T(\Delta S_{conf}^{rs}) = \min(\Delta G_{RR}^{TS}) - \min(\Delta G_{RR}^{RS}) + T\Delta S_{cage}^{RS} \quad (1)$$

Here, ΔG_{RR} denotes the restraint release free energy at the RS and TS respectively. It should be noted that, per definition, these free energies also include an enthalpic contribution. However, it has been demonstrated⁸¹ that this enthalpic contribution is minimized by the restraint coordinates that give the lowest RR free energy. Therefore, this procedure was repeated multiple times, sampling over several different starting coordinates, in order to identify the restraint coordinates that minimized ΔG_{RR} in both RS and TS (this is denoted by $\min(\Delta G_{RR})$ in Eq. 1). ΔS_{cage} is the entropy associated with applying a special restraint ($K_{cage}=0.3 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) to the oxygen atom of the nucleophile and the phosphorus or sulfur atom (as relevant) of the substrate, in order to keep them at a specified contact distance, thus bringing the reacting fragments from a molar volume to the same solvent cage (see the discussion in Ref. ⁸¹). Finally, in order to save computational time, Eq. 1 can be replaced by:

$$-T\Delta S_{conf} = -T\Delta S(K = K_1)_{QH} + \min[\Delta G_{RR}(K = K_1 \rightarrow K = 0)] \quad (2)$$

Here, $T\Delta S(K = K_1)_{QH}$ denotes the entropy obtained by the quasiharmonic approximation⁸³, where K_1 is the initial value of the restraint. Despite known problems with this approximation⁸⁴, it nevertheless tends to be valid when the restraints are significant, and only becomes problematic when they become small, which results in a range of very shallow anharmonic potential energy surfaces. Therefore, it is possible to combine the QH and RR approaches and evaluate the *absolute* entropy while applying a strong restraint (in this case $10 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) using the QH approximation, and then evaluate the free energy associated with releasing this restraint using the RR approach, and add it as a correction. This approach has been successfully applied to not only the ribosome⁸², but also (and perhaps more relevantly

to this work), to numerous studies of phosphate hydrolysis (e.g. Refs. ^{18,57}). Both RR and QH calculations were performed at the RS and TS using a 16Å simulation sphere of explicit water molecules subject to the Surface Constrained All Atom Solvent (SCAAS) boundary conditions⁸⁵, using an EVB potential that approximates the *ab initio* surface, as in our previous studies. The QH calculations were performed using $K_1=10 \text{ kcal mol}^{-1} \text{ Å}^{-2}$, and the RR-FEP calculations were subsequently performed while changing K_1 from 10 to 0.0 $\text{kcal mol}^{-1} \text{ Å}^{-1}$, in 41 mapping steps, each of which was 3ps in length, using a 1fs time step, at 300K. The minimum value of ΔG_{RR} was then obtained from 10 series of runs. All QH and RR calculations were performed using the MOLARIS software package, and the ENZYMIX forcefield^{85,86}, and the calculated configurational entropy was added as a correction to the energetics of the *ab initio* TS, so that the resulting activation barriers also take the solute configurational entropy into account. Finally, in order to evaluate the total activation entropy, we also estimated $T\Delta S_{solv}^{\ddagger}$ using the Langevin dipoles solvation model (see also Ref. ⁵⁷), and demonstrate that we are able to reproduce the experimentally observed trend with high accuracy, as was also the case in our previous study⁵⁷.

III. RESULTS AND DISCUSSION

III.1 FREE ENERGY SURFACE FOR *p*-NITROPHENYL PHOSPHATE HYDROLYSIS

The first reaction to be examined is water attack on the *p*-nitrophenyl phosphate dianion (pNPP, see Fig. 1 which presents a comparison of postulated mechanisms for the hydrolysis of the pNPP dianion, as well as that of the analogous sulfate monoester monoanion). This system is important as it serves as a prototype for studying phosphoryl transfer, both in enzymes and in solution^{2,3,33,56,87}. The measured rate⁷ for the aqueous hydrolysis of pNPP at 39° C is $1.6 \times 10^{-8} \text{ s}^{-1}$, which corresponds to an activation free energy of 29.5 kcal/mol from transition state theory. It has been argued based on experimental

markers^{7,33,68,88} such as isotope effect measurements and linear free energy relationships that this reaction will proceed *via* a concerted A_ND_N mechanism, with little nucleophile participation and a largely broken bond to the leaving group in the transition state (and in one case for the full on intervention of a metaphosphate species⁸⁹). However, extensive examination and computational studies of phosphoryl transfer reactions have demonstrated that in the case of such complex reactions (where multiple possible mechanisms can be equally viable for the same system), experimental data needs to be approached with care and cannot unambiguously determine a single mechanism^{14-18,21,24,57,58}.

Interestingly, despite the importance of pNPP as an experimental marker to examine phosphoryl transfer, to the best of our knowledge, there are very few^{79,90} computational studies available in the literature that directly examines its reactivity. However, a study of the alkaline hydrolysis of pNPP⁷⁹, Guo and coworkers set up a system involving a hydroxide ion, pNPP, and $n=0, 12, 13$, and 14 explicit water molecules, and found that the nucleophile is actually a water molecule, with the hydroxide anion acting as a general base. This would be in line with the postulated resistance of phosphate monoester dianions to hydroxide attack⁷. The authors then find that the hydrolysis proceeds *via* an expansive transition state, with P-O distances of 2.65 and 2.75 Å to the incoming nucleophile and departing leaving group respectively. Such an expansive transition state could be expected from the attack of an anionic species on a dianion, and we obtained a similarly expansive transition state when attempting to examine hydroxide attack on phenyl phosphate¹⁸. Additionally, a relatively expansive transition state (P-O and P-N distances of 2.237 and 2.395 Å respectively) was obtained for the corresponding aminolysis reaction⁹⁰, using a quantum mechanical/effective fragment approach.

Fig. 2 shows the calculated free energy surface for water attack on pNPP, as well as the geometry of a corresponding transition state structure, obtained by performing an unconstrained transition state optimization on one of the relevant structures on the surface. That is, from following the contours on this surface from the reactant to product regions, it appears that the reaction is proceeding through a concerted A_ND_N mechanism with a single transition state, with a high-energy ridge separating the

reactant and product states. Note however that the process being examined here is a complex process in which, as discussed in Section II.2, the nucleophilic attack is potentially coupled with a proton transfer to the substrate, which in turn increases the complexity of the energy surface (as a reminder, the surfaces presented in this work are projections onto two dimensions, and as such are very sensitive to the correct choice of reaction coordinate). Therefore, even though the strategy of not explicitly defining the proton transfer as a separate coordinate has been successful in analogous systems, such as in ^{16,57}, this is not always guaranteed to be the case. Additionally, while the purpose of examining the 2-D free energy surface as opposed to isolated transition states is to also examine the overall pathway, the topology of the calculated free energy surface for *p*-nitrophenyl phosphate hydrolysis does not make it immediately clear where the actual transition state lies along this ridge (in contrast to the surface for *p*-nitrophenyl sulfate hydrolysis, where a transition state can be easily marked on the surface, as shown in Section III.2). Therefore, in order to be able to further examine the reaction mechanism, we have followed the contours of the plot, taken a high-energy point along the ridge, and performed an unconstrained transition state optimization on this point, to see if it will “relax” to a relevant transition state. This resulted in a transition state with P-O distances of 1.86 and 2.23 Å to the leaving group and nucleophile respectively (see Fig. 2). Therefore, while the surface shown in Fig. 2 can give a rough guideline as to the nature of the pathway (i.e. $A_N D_N$ vs. $A_N + D_N$), the actual relaxed $A_N D_N$ transition state falls off the ridge shown in Fig. 2 (even though it is still in the *approximate* region marked by the question mark). A gas-phase frequency calculation on this structure yields a single imaginary frequency (244 cm⁻¹) corresponding to motion along O_{nuc}-P-O_{lg} (i.e. P-O formation/cleavage) with concomitant proton transfer from O_{nuc} to a non-bridging oxygen on pNPP. However, as the transition state optimization was performed using the continuum model (see Section II.2), and it is unclear how reliable the solute vibrational frequencies obtained from the continuum model actually are (see discussion in Ref. ¹⁷ and references cited therein), therefore, we also followed the intrinsic reaction coordinate (IRC) in both the reactant and product directions to further characterize the transition state. The IRC calculations were

performed in 50 steps in each direction, after which the resulting structures were subjected to unconstrained geometry optimizations. By doing this, it was possible to obtain an unconstrained reactant complex, however, attempting to optimize the structure obtained after following the IRC in the product direction (which resembles a product complex in which the P-O_{nuc} bond has been formed and the P-O_{lg} bond is essentially cleaved) without constraints resulted in this structure “exploding” (i.e. the two fragments flew away from each other), which is again most likely a simulation artefact due to the relatively high charge on the system. Nevertheless, from following the IRC, it is clear that the system is moving towards a product complex and not a pentavalent intermediate. Additionally, note that the proton on the non-bridging oxygen atom of the phosphate in the transition state has been transferred back to the attacking water molecule in the reactant complex, but has *not* been transferred to the leaving group in the product complex (see the discussion below). This is also in agreement with examining the individual structures comprising the surface shown in Fig. 2, where structures in the reactant region correspond to a water molecule and the pNPP dianion, structures along the ridge in the approximate transition state region correspond to hydroxide anion and the pNPP monoanion, and structures in the product region correspond to a phosphate monoanion and p-nitrophenyl. Cartesian coordinates for the optimized transition state as well as the structures obtained from following the IRC and finally the optimized reactant complex are provided in the Supporting Information. Note also that the optimized transition state obtained for *p*-nitrophenyl phosphate hydrolysis is very similar to but slightly more expansive than the approximate transition state obtained by Warshel and coworkers for water attack on the phenyl phosphate dianion¹⁶, which is what would be expected from the more acidic leaving group of pNPP (7.0 vs. 10.0 for PhO⁻). Finally, the structure presented in Fig. 2, while not completely compact, is nevertheless still in contrast with the traditional interpretation of the experimental data which has described the transition state as generally loose, and even metaphosphate-like⁸⁹. However, it has been demonstrated that it can be quite challenging to make conclusive mechanistic assignments based on experimental data alone (i.e. without simulations that can reproduce the data), as not only can there be

multiple equally viable mechanisms for the same system (see e.g. ^{16,17,24,57}), but also, different mechanistic possibilities can give rise to identical experimental markers (see discussion in ^{14,16,17,57}), further emphasizing the importance of combining theory and experiment in studies of reaction mechanisms. Regardless, coming back to the present case, the calculated activation barrier of 33.0 kcal/mol is within 3.5 kcal/mol of the experimental value⁷ (29.5 kcal/mol), and is thus in acceptable agreement with the experimental value. The energy decomposition for $\Delta g^\ddagger_{\text{calc}}$ for the optimized transition state for pNPP hydrolysis is shown in Table 1. From this table, it can be seen that the solvent destabilizes the transition state. This is similar to the results of Refs. ^{79,90}, which both found that in the absence of solvent, the isolated pNPP dianion spontaneously dissociated to metaphosphate and *p*-nitrophenoxide (though, as mentioned in the Computational Details, we believe that this is most likely rather a simulation artifact when dealing with such highly charged systems), whereas the inclusion of water molecules resulted in a barrier to the reaction. This is also similar to our previous studies of phosphate monoester dianions⁵⁷.

Figure 1: Potential mechanisms for (a) *p*-nitrophenyl phosphate and (b) *p*-nitrophenyl sulfate hydrolysis. In both cases, the most likely pathway is an A_ND_N mechanism proceeding through a single, concerted transition state, which can be either compact or expansive in nature. Note that while it could indeed be protonated, *p*-nitrophenol is a sufficiently good leaving group that protonation is not *necessarily*

required for leaving group departure (and, in fact, experimentally measured kinetic isotope effects suggest that it is not³³).

Figure 2: Free energy surface (kcal mol^{-1}) and optimized transition state for water attack on *p*-nitrophenyl phosphate. From the surface, it can be seen that the reaction proceeds *via* a concerted A_ND_N transition state, passing over a high-energy ridge separating the reactant and product states. However, the precise location of the transition state along this ridge is unclear from the surface alone, as indicated by the question mark on the surface.

Table 1: Energy decomposition of $\Delta g^\ddagger_{\text{calc}}$ for the hydrolysis of *p*-nitrophenyl phosphate (pNPP) and *p*-nitrophenyl sulfate (pNPS). All energies are given in kcal/mol.

Species ^a	ΔE_{gas}	ΔE_{pol}	$\Delta\Delta G_{\text{solv}}$	ZPE	$-T\Delta S_{\text{conf}}$	$\Delta g^\ddagger_{\text{calc}}$	$\Delta g^\ddagger_{\text{exp}}$
pNPP	11.5	-2.5	19.0	1.5	3.5	33.0	29.5
pNPS	23.9	0.5	-1.3	1.6	8.5	33.2	30.4

Finally, it is possible to use the ^{15}k secondary isotope effect to measure the negative charge delocalization into the nitro group of the *p*-nitrophenol, and thus to determine whether the leaving group is departing as the anion or if some (or all) of the negative charge resulting from P-O bond fission has

been neutralized as a result of protonation of the leaving group³³. The experimentally measured ¹⁵k isotope effect is 1.003, suggesting no protonation of the leaving group⁹¹. This is to be expected considering the pK_a of *p*-nitrophenol (~7), which does not necessarily require protonation to depart (though, considering the rates of phosphate monoester hydrolysis⁸, this is likely to be as much a function of pH as of pK_a). However, it is worth mentioning that our calculated structures also agree with this observation. That is, as can be seen from Fig. 2, in the transition state, a proton has been transferred from the attacking water molecule to one of the non-bridging oxygen atoms of the phosphate in a substrate-as-base mechanism, ultimately resulting in hydroxide attack on a protonated phosphate monoanion (though this proton transfer is concerted with the phosphoryl transfer in our simulation setup). It should be borne in mind that no extra constraints were placed on this proton, and therefore this is not a “forced” proton transfer. It is also worth noting that once a proton has been transferred to the phosphate to generate a phosphate monoanion, the substrate has the same overall charge (-1) as its sulfate analogue, though what one is now observing is hydroxide rather than water attack on a monoanion, so one could still expect the chemistry to be potentially different from the corresponding water attack on pNPS. In any case, as mentioned above, even at the extremities of the surface (*i.e.* extended P-O_{lg} distances), no protonation of the leaving group is observed and the leaving group departs as an anion, in good agreement with the qualitative interpretation expected from the ¹⁵k isotope effect.

III.2 FREE ENERGY SURFACE FOR *P*-NITROPHENYL SULFATE HYDROLYSIS

As mentioned in the introduction, in contrast to the vast body of literature available on phosphoryl transfer reactions, much less effort has been invested into studying sulfuranyl transfer, despite its almost equally central biological role. However, there have been elegant studies that directly compare phosphoryl and sulfuranyl transfer, using in particular isotope effects as probes for the reaction mechanism^{33,68}. The measured rate of hydrolysis of the *p*-nitrophenyl sulfate monoanion in the pH

independent region is $1.87 \times 10^{-9} \text{ s}^{-1}$ at 35° C^{68} , which gives an activation barrier of 30.4 kcal/mol from transition state theory, in comparison to 29.5 kcal/mol for pNPP hydrolysis. The measured ^{18}k (oxygen) and ^{15}k (nitrogen) isotope effects⁶⁸ for pNPP hydrolysis and pNPS hydrolysis are very similar, suggesting similar mechanisms. Specifically, once again, the ^{15}k isotope effect of 1.0026 would suggest that the leaving group is departing as an anion with a near full negative charge in the transition state (which has also been suggested to be indicative of a large degree of bond cleavage to the leaving group in the transition state), and the $^{18}\text{k}_{\text{nonbridge}}$ isotope effect (i.e. the ^{18}O isotope effect on the non-bridging oxygens of pNPS) has been presented as evidence that the sulfonyl group resembles SO_3 in the transition state. However, as mentioned earlier, the activation entropy of -18.5 eu for pNPS hydrolysis is significantly different than that from pNPP hydrolysis, and the origin for this difference in activation entropy has not been rationalized.

Figure 3: Free energy surface (kcal mol⁻¹) and approximate transition state for water attack on *p*-nitrophenyl sulfate.

Fig. 3 shows our calculated surface for the hydrolysis of the pNPS monoanion as well as the geometry of the corresponding transition state. As can be seen from this figure, the reaction proceeds through a concerted $\text{A}_{\text{N}}\text{D}_{\text{N}}$ mechanism, with concomitant nucleophilic attack and leaving group departure, as was

also the case for pNPP hydrolysis. However, this is where the similarities between the two systems end. That is, while pNPP hydrolysis appears to follow a (in comparison) quite compact reaction pathway with far greater bond formation to the incoming nucleophile than bond cleavage to the leaving group in the transition state, the surface for pNPS hydrolysis gives a qualitatively very different picture. As with pNPP, the departing leaving group is an anion, in good agreement with the measured KIE⁶⁸. Additionally, the sulfonyl group in flight does indeed resemble SO₃, as would be expected from the measured isotope effect. However, with S-O distances of 2.2 and 2.5 Å to the incoming nucleophile and departing leaving group respectively, the transition state for the sulfonyl transfer reaction is noticeably more expansive than the transition state for the analogous phosphoryl transfer reaction, and it would appear that no proton transfer from the attacking nucleophile has occurred yet. The lack of proton transfer to the SO₃ moiety at this stage can be easily rationalized based on the fact that there is an impetus for protonating the highly charged pNPP dianion prior to nucleophilic attack, which is reduced due to the fact that pNPS is a monoanion. However, the fact that the sulfonyl transfer reaction would appear to proceed *via* a more expansive transition state than the analogous phosphoryl transfer is somewhat surprising, though again, this could be a result of the fact that the charge on the dianionic “phosphorane”-like species has been reduced by the proton transfer from the attacking nucleophile, making such a compact transition state less unfavourable. Additionally, note that unlike the calculated surface for pNPP hydrolysis (Fig. 2), in the case of pNPS hydrolysis, it is very easy to clearly mark an approximate transition state on the surface, possibly as a result of the fact that the lack of proton transfer to the sulfate upon approaching the transition state results renders the surface less complex with more easily definable reaction coordinates.

The energy decomposition of $\Delta g^{\ddagger}_{\text{calc}}$ for pNPS hydrolysis is shown in Table 1. From this table, it can be seen that our calculated activation barrier of 33.2 kcal/mol is, again, within acceptable agreement of the experimental value of 30.4 kcal/mol (i.e. within 3 kcal/mol). However, even though the overall $\Delta g^{\ddagger}_{\text{calc}}$ for pNPS hydrolysis is similar to that for pNPP hydrolysis, the energy decomposition is quite

different. That is, while the hydrolysis of pNPP proceeds with significant solvent destabilization (compared to the gas-phase energetics of the same structure), at -1.4 kcal/mol, the solvation effect in the case of pNPS hydrolysis is minimal. This is important, as it has been repeatedly demonstrated that the major contributor to the catalytic power of enzymes is electrostatic preorganization^{49,92,93}, with the enzyme providing an environment that stabilizes the transition state of the reaction far better than water does. Clearly, if the solvation effects for the hydrolysis of pNPP and pNPS are significantly different, this means that the preorganization requirements at the active site for efficient catalysis are also quite different, and it raises the interesting question of how an active site that is preorganized for the hydrolysis of one class of substrate can nevertheless efficiently catalyze the other.

III.3 COMPARING ACTIVATION ENTROPIES FOR *P*-NITROPHENYL PHOSPHATE AND *P*-NITROPHENYL SULFATE HYDROLYSES

The energy decompositions shown in Table 1 only show the solute and not the solvent contribution to the overall activation entropy, as the latter contribution is implicitly included in the solvation model. However, in order to quantify this contribution, we have estimated the solvation entropy using the Langevin dipoles solvation model, as was done in our previous work⁵⁷, and the resulting total activation entropies for both pNPP and pNPS hydrolyses are shown in Table 2. From this table, it can be seen that, in both cases, the calculated activation entropies are slightly lower than (but nevertheless in good agreement with) the experimental values, at -1.9 and 4.3 kcal/mol compared to the experimental values of -1.1 and 5.1 kcal/mol for pNPP and pNPS hydrolysis respectively. However, even more importantly, it is worth emphasizing that the calculations correctly reproduce the *trend* in the activation entropies, with the activation entropy for pNPP hydrolysis being significantly smaller than that for pNPS hydrolysis, despite the latter proceeding through a comparatively more compact transition state.

Table 2: Energy decomposition of ΔS_{tot} for the hydrolysis of *p*-nitrophenyl phosphate (pNPP) and *p*-nitrophenyl sulfate (pNPS). All energies are given in kcal mol⁻¹.

Species ^a	$-T\Delta S_{\text{solv}}$	$-T\Delta S_{\text{conf}}$	$-T\Delta S_{\text{tot,calc}}$	$-T\Delta S_{\text{tot,exp}}$

pNPP	-5.4	3.5	-1.9	-1.1
pNPS	-4.2	8.5	4.3	5.1

This is of interest, as it has traditionally been argued that the small entropy of activation for the hydrolysis of phosphate monoester dianions is supportive of a dissociative (expansive) transition state⁷, whereas, as can be seen from Figs. 2 and 3, pNPP hydrolysis would appear to proceed through a more compact transition state than pNPS hydrolysis despite having a significantly smaller activation entropy. However, we demonstrated in our earlier study⁵⁷ that both compact and expansive transition states can have similar entropies of activation. Additionally, as mentioned above, we have correctly reproduced the *trend* in the activation entropies, with the calculated activation entropy for pNPS hydrolysis being larger than that for pNPP hydrolysis by 6.2 kcal/mol (which is a quite significant difference). This follows on from our earlier study⁵⁷, in which we were able to reproduce the experimental activation entropies for the hydrolyses of both the methyl phosphate dianion and the methyl pyrophosphate trianion with similarly high accuracy. However, while the purpose of our earlier work was to explicitly explore whether compact (associative) and expansive (dissociative) transition states have significantly different activation entropies, the present work specifically focuses on a comparative study of the reactivity of two analogous but chemically different monoesters, which can both be catalyzed by the same enzyme in many cases, and we demonstrate that the two reactions have in fact quite different bond orders to the incoming nucleophile and departing leaving group at the transition state, with very different solvation effects. As far as the activation entropies are concerned, our interest was in seeing whether the unexpectedly large difference between the two monoesters could be reproduced (which is indeed the case). The fact that the trend in the “size” of the transition state (i.e. compact vs. expansive) is the opposite of that which one would expect from the traditional mechanistic interpretation of activation entropies further emphasizes our earlier argument^{14,57} that the interpretation of activation entropies in terms of bond orders is overly simplified, as the activation entropy also depends on other factors such as

solvation and desolvation effects associated with charge redistribution upon approaching the transition state, entropy changes associated with intramolecular degrees of freedom as the transition state is approached, and steric factors determining the configurational volumes available to reactants during the reaction, to name a few examples, making it difficult to determine the precise origin of such differences in activation entropies for different reactions, or to use activation entropies as determinants of mechanism. Note however that the centrality of solvation effects in determining the activation entropy has been also highlighted by others⁹⁴, and while we are not sure what the precise origin of the difference in activation entropy is (other than that it is not directly correlated to bond orders), the possibility that it arises out of differences in solvation effects for the two reactions (as is seen in Table 1) has already been considered⁶⁸, and this may well be the best rationalization for the difference in the two activation entropies (though at this stage any conclusion beyond the fact that the trend in the activation entropies in this particular case is not driven by bond order at the transition state would still be speculative).

III.4 A COMPARISON OF CALCULATED AND EXPERIMENTAL KINETIC ISOTOPE EFFECTS FOR *P*-NITROPHENYL PHOSPHATE AND *P*-NITROPHENYL SULFATE HYDROLYSES

Experimentally measured isotope effects are a powerful and versatile component of the physical organic chemistry “toolbox”⁹⁵, and have been used extensively as probes of both phosphoryl and sulfonyl transfer reactions (see discussion in e.g. ^{2,33,96-98}, and references cited therein), as well as countless other systems (e.g. ⁹⁹⁻¹⁰²). However, as with the experimentally measured activation entropies, it has been proposed that the qualitative interpretation of kinetic isotope effects in terms of degrees of bond fission should be approached with care in the case of phosphoryl transfer reactions^{14,16}, as both compact (associative) and expansive (dissociative) transition states can give rise to similar effects, making the mechanistic assignment not necessarily unique. Nevertheless, and relevantly to the present work, there have been elegant experimental studies^{33,56,68} that have characterized the kinetic isotope

effects for both *p*-nitrophenyl phosphate and sulfate hydrolysis, and specifically, it was observed that the experimentally measured kinetic isotope effect for the hydrolysis of the aryl sulfate monoester is very

Figure 4: A schematic diagram of the positions of the experimentally measured isotope effects being examined in this work for *p*-nitrophenyl phosphate. Note that the isotopic substitution positions for *p*-nitrophenyl sulfate are analogous.

similar to that of its aryl phosphate counterpart. Therefore, both the reaction rates and isotope effects for the two reactions are very similar, and the only “outlier” is the corresponding activation entropies, the trend in which was demonstrated to be reproducible in Section III.3. The question therefore becomes that of whether (at least the trend in) the experimentally observed kinetic isotope effects are also reproducible, despite the fact that the transition states for the phosphoryl and sulfuryl transfer reactions appear to vary significantly in terms of bond orders to the incoming nucleophile and departing leaving group.

Fig. 4 shows a diagram of the positions at which isotope effects have been measured for *p*-nitrophenyl phosphate hydrolysis (note that the analogous isotope effects have also been measured for *p*-nitrophenyl sulfate hydrolysis), and the corresponding calculated and experimental values are shown in Table 3. The qualitative interpretation of the ^{15}k isotope effect in terms of protonation of the leaving

group has already been discussed in sections III.1 and III.2. The $^{18}\text{k}_{\text{bridge}}$ and $^{18}\text{k}_{\text{nonbridge}}$ isotope effects are primary and secondary isotope effects respectively, as the S-O_{bridge} bond is cleaved in the transition state whereas the S-O_{nonbridge} bonds are not. The similar $^{18}\text{k}_{\text{bridge}}$ values of 1.0189 and 1.0210 for the phosphoryl

Table 3: A comparison of the calculated and experimentally observed ^{15}k , $^{18}\text{k}_{\text{bridge}}$ and $^{18}\text{k}_{\text{nonbridge}}$ kinetic isotope effects (KIE) for *p*-nitrophenyl phosphate (pNPP) and sulfate (pNPS) hydrolysis.

KIE ^a	pNPP ^b		pNPS ^c	
	Calculated	Observed	Calculated	Observed
^{15}k	1.0004	1.0028	1.0019	1.0026
$^{18}\text{k}_{\text{bridge}}$	1.0002	1.0189	1.0208	1.0210
$^{18}\text{k}_{\text{nonbridge}}$	0.9804	0.9994	0.9993	0.9951

^a For a definition of the substitution position for each isotope effect, see Fig. 6. ^b Experimentally measured at pH 10.0 and 85° C. Experimental data obtained from Refs. ^{56,68}. pNPP denotes the optimized transition state shown in Fig. 2. ^d Experimentally measured at pH 9.0 and 95° C. Experimental data obtained from Ref. ⁶⁸. Calculation performed on the approximate transition state from the surface shown in Fig. 3. Note that the calculations of the vibrational frequencies were performed so as to match the relevant temperature for the corresponding experimentally measured KIE.

and sulfuryl transfers have been used (in combination with the similar ^{15}k effect) to argue for the two reactions proceeding with very similar transition states in which the bond to the leaving group is largely cleaved in the transition state⁶⁸. Finally, it has been argued^{33,103} that the $^{18}\text{k}_{\text{nonbridge}}$ isotope effect is small and inverse for expansive (dissociative) transition states with a large degree of bond cleavage to the

leaving group in the transition state, and large and normal for reactions in which the transition state is much more compact (associative).

In order to investigate this further, the ^{15}k , $^{18}k_{\text{bridge}}$ and $^{18}k_{\text{nonbridge}}$ isotope effects have been calculated from the differences in gas-phase zero-point energies between the ground state and transition state with the different relevant isotope compositions (as was also done in e.g. ¹⁶), using the Gaussian03 thermochemistry module, the 6-311+G** basis set and the B3LYP density functional. All calculations were performed on the relevant transition states, the coordinates for which are presented in the Supporting Information, relative to a relevant “reactant complex” (obtained by performing unconstrained optimizations on either the structure obtained from following the IRC for pNPP hydrolysis, or a low energy point in the reactant region of the surface shown in Fig. 3 for pNPS hydrolysis). The resulting calculated KIE (with the corresponding experimental values for comparison) are shown in Table 3. As can be seen from this Table, in the case of pNPS hydrolysis, both the calculated ^{15}k and $^{18}k_{\text{bridge}}$ KIE are in good qualitative and quantitative agreement with experiment. Additionally, while there is larger deviation between the calculated and experimental $^{18}k_{\text{nonbridge}}$ KIE, however, the calculated inverse $^{18}k_{\text{nonbridge}}$ is at least in qualitative agreement with the experimental value.

The situation for pNPP hydrolysis is more complex. The experimentally measured KIE for both pNPP and pNPS hydrolysis are very similar, as shown in Table 3. However, while qualitatively in the right direction, there are comparatively larger deviations from experiment for both the calculated ^{15}k and $^{18}k_{\text{bridge}}$ KIE for pNPP hydrolysis, compared to those for pNPS hydrolysis. The upper limits expected for these KIE are ^{15}k and $^{18}k_{\text{bridge}}$ values of 1.003 and 1.03 for a more expansive (“loose”) transition state with extensive bond cleavage to the leaving group, and no charge neutralization of the leaving group. Correspondingly, smaller values of ~1.000 are expected for both the ^{15}k and $^{18}k_{\text{bridge}}$ KIE for a more compact (“tight”) transition state with little to no bond fission to the leaving group (see the discussion in e.g. Ref. ³³). The sensitivity of the calculated KIE to structure have been examined extensively

elsewhere (e.g. ¹⁰⁰). Nevertheless, interestingly, both the ¹⁵k and ¹⁸k_{bridge} values are much smaller than the experimentally measured values, and qualitatively closer to what one would expect for a compact transition state (see discussion in e.g. ³³). On the other hand, both structures yield an inverse ¹⁸k_{nonbridge} KIE, in agreement with experiment, and in contrast to the traditional interpretation of an inverse ¹⁸k_{nonbridge} KIE representing a metaphosphate-like transition state with extensive bond cleavage to the leaving group (again, see discussion in e.g. Ref. ³³). However, it should be borne in mind that the approach used here to calculate the KIE is relatively simplistic, and, in theory, better agreement with experiment could have been obtained by use of a far more elaborate approach such as e.g. a centroid path integral method^{104,105} (and, in particular, for instance the robust quantized classical path approach¹⁰⁶) should be used. Additionally, it has been demonstrated that at least in the case of equilibrium isotope effects (EIE) in phosphate deprotonation reactions¹⁰⁷, the calculated EIE are greatly sensitive to microscopic solvent effects, with the addition of a progressively increasing number of explicit water molecules in the first solvation shell significantly affecting the calculated EIE, and it is highly likely that this may also apply to the calculated KIE (note that in both cases, the calculations are complicated by the proton transfer to the phosphate). Finally, it is worth pointing out that while it is clearly important that the theoretically proposed transition states can produce KIE that are at least in plausible agreement with the experimentally observed values, the *qualitative* analysis of the experimentally measured KIE should be approached with care. That is, as early as 1955, Bigeleisen and Wolfsberg¹⁰⁸ demonstrated that in the simple H₂/HT+Cl reaction, discrepancies between the theoretical and experimental signs of the activation energy difference in the KIE could not be removed by simple geometric changes (as it was possible to construct both linear and bent transition states that were in reasonable agreement with all the known isotope effects, see also additional discussion in ⁹⁵). More recently (and relevantly to the present work), Warshel and coworkers also demonstrated that the qualitative interpretation of experimental KIE for phosphoryl transfer reactions is complex (particularly when proton transfers are involved), and that associative and dissociative transition states can give rise

to similar KIE (see discussion in Refs. ^{14,16}). However, when approached rigorously, a combination of theory and experiment can give important insights into transition state geometries from measured KIE (e.g. ¹⁰⁰). Additionally, as can be seen from Table 3, while qualitatively interpreting experimental KIE in terms of degrees of bond fission at the transition state appears to be a nontrivial issue, the calculated KIE on the optimized and approximate transition structures obtained from the surfaces shown in Figs. 2 and 3 respectively are at least in plausible agreement with the experimentally measured values.

IV. CONCLUSIONS

The present work presents a comparative theoretical investigation of the hydrolysis of *p*-nitrophenyl sulfate and *p*-nitrophenyl phosphate in aqueous solution. Based on the available experimental evidence, it is expected that the two reactions will proceed through transition states with similar geometries and mechanisms^{33,68}, and the overall observed activation barriers are also quite similar^{7,68}. Interestingly, however, despite the perceived similarities between the two reactions, they proceed with very different activation entropies. In light of this, and the comparative lack of theoretical studies of sulfuryl transfer in aqueous solution in the literature, we have examined 2-dimensional free energy surfaces for both reactions, as well as calculating both the solute and solvent contributions to the overall activation entropy and the experimentally measured ¹⁵N and ¹⁸O kinetic isotope effects. We are able to reproduce both the experimentally observed activation barriers with reasonable accuracy (i.e. within ~3.5 kcal/mol), and, more importantly, and we are also able to correctly reproduce the trend in the overall activation entropies, as well as obtaining plausible agreement between theory and experiment for the relevant KIEs. We observe that while the overall activation barriers for pNPP and pNPS hydrolysis are quite similar, both the nature of the transition state and the energy decomposition of the calculated activation barriers are quite different. That is, while it appears that pNPP hydrolysis proceeds *via* a

comparatively fairly compact transition state with significant solvent destabilization, pNPS hydrolysis proceeds *via* a far more expansive transition state with a much smaller solvent effect. This is interesting in light of the fact that several members of the alkaline phosphatase superfamily can cross-catalyze both phosphoryl and sulfuryl transfer⁵⁰, and yet such fundamental differences in the nature of the transition state and the solvation requirements would mean that the enzyme active site would have to have quite different electrostatic preorganization in order to efficiently catalyze either reaction. Therefore, it is perhaps unsurprising that such enzymes show far less proficiency towards their promiscuous rather than their native substrates (since the enzyme has not been “set up” to optimally catalyze the promiscuous reaction(s)), but, equally, it raises a question as to how they are able to catalyze substrates with such different requirements for efficient catalysis in the first place. There have been computational studies of catalytic promiscuity in unrelated enzymes¹⁰⁹, which have used careful energy-based considerations to explore what the origin of the catalytic effect for the different substrates actually is. However, in most cases where catalytic promiscuity is observed, the enzyme involved catalyzes the reactions of quite different substrates, whereas in the case of the alkaline phosphatase superfamily, the relevant enzymes appear to be able to discriminate between substrates where the difference between the two substrates is minute (e.g. replacing phosphorus for sulfur). Computational studies in our group are currently underway to examine the origin of such phosphate/sulfate cross-promiscuity, and we believe that understanding how enzymes are able to catalyze substrates with such different catalytic requirements from their native substrate is a major step towards understanding evolution of function within enzyme superfamilies.

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SUPPORTING INFORMATION AVAILABLE

Supporting information. Cartesian coordinates of relevant stationary points for both *p*-nitrophenyl phosphate and sulfate hydrolysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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