Published in final edited form as:

Biochim Biophys Acta. 2015 November ; 1854(11): 1768–1775. doi:10.1016/j.bbapap.2015.03.010.

Kinetic Isotope Effects In The Characterization Of Catalysis by Protein Tyrosine Phosphatases

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Abstract

Although thermodynamically favorable, the uncatalyzed hydrolysis of phosphate monoesters is extraordinarily slow, making phosphatases among the most catalytically efficient enzymes known. Protein-tyrosine phosphatases (PTPs) are ubiquitous in biology, and kinetic isotope effects were one of the key mechanistic tools used to discern molecular details of their catalytic mechanism and the transition state for phosphoryl transfer. Later, the unique level of detail KIEs provided led to deeper questions about the potential role of protein motions in PTP catalysis. The recent discovery that such motions are responsible for different catalytic rates between PTPs arose from questions originating from KIE data showing that the transition states and chemical mechanisms are identical, combined with structural data demonstrating superimposable active sites. KIEs also reveal perturbations to the transition state as mutations are made to residues directly involved in chemistry, and to residues that affect protein motions essential for catalysis.

Keywords

Protein-tyrosine phosphatase; Phosphoryl transfer; Kinetic isotope effect; Protein dynamics

1. Introduction

The hydrolysis of a phosphate ester is a simple reaction: ester plus water yields inorganic phosphate and the ester alcohol. This reaction is ubiquitous in biology, and in 1987 the late Frank Westheimer wrote a *Science* article[1] entitled “Why Nature Chose Phosphates.” Phosphate esters have a number of unique properties that suit them to their many biological roles, including serving as stable linkers in nucleic acids, in energy rich molecules like ATP, and a host of phosphorylated intermediates in metabolism. Furthermore, nature uses the yin-yang system of kinases and phosphatases to regulate many biological processes by controlling the phosphorylation level of proteins. Although a simple reaction, and thermodynamically favorable, the hydrolysis of a phosphate ester has a very high activation barrier. In fact, the uncatalyzed hydrolysis of phosphate monoesters in the dianion state, the substrate of most phosphatases, is one of the slowest known reactions of biological

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relevance [2]. This makes phosphatases among the most catalytically proficient enzymes known. As a result, considerable effort has been devoted to the study of the mechanisms of the uncatalyzed hydrolysis reactions of all three types of phosphate esters (monoesters, diesters, and triesters) and their enzymatic counterparts. A number of fine reviews of this field have appeared over the years, [3–8] and a particularly comprehensive one (as well as the most recent) by Lassila et al. [9] excellently summarizes the key questions and the major experimental methods that have been used to address them.

A major mechanistic question for uncatalyzed phosphoryl transfer reactions was whether the reactions are concerted, or proceed through stable intermediates. A related issue was the nature of the transition states, a question that KIEs are well suited to address. Given the dramatic rate accelerations imparted by phosphatases, it was important to learn whether these enzymes altered the transition states from those of uncatalyzed reactions in solution. The Cleland lab was a pioneer in the application of isotope effects to enzymology. In the late 1980s a method to measure heavy-atom isotope effects on phosphoryl transfer reactions was developed that was applied to uncatalyzed reactions, and to the then-recently discovered and characterized protein-tyrosine phosphatases (PTPs). This article focuses on how kinetic isotope effects (KIEs) were used to address fundamental mechanistic questions regarding uncatalyzed phosphomonoester reactions, and their hydrolysis by PTPs.

2. Mechanistic questions for phosphoryl transfer

2.1 Concerted or stepwise reactions for uncatalyzed reactions

One of the longest-standing questions in the field was whether phosphoryl-transfer reactions are concerted, or occur stepwise with intermediates. A More O'Ferrall-Jencks diagram in Figure 1 depicts the continuum of potential transition states for the hydrolysis of a monoester. Concerted reactions have transition states within the box, and stepwise pathways follow one of the boundaries. A concerted reaction with a transition state in which the bond to the leaving group has largely broken but nucleophilic involvement is small is termed a loose transition state, and sometimes called a dissociative transition state. The use of the latter term can confuse the issue by failing to distinguish between a dissociative *mechanism*, in which a metaphosphate intermediate is formed. This pathway follows the bottom axis all the way to the right corner. Metaphosphate is expected to be a highly reactive intermediate, rapidly attacked by a nucleophile such as water. The opposite scenario, in which bond formation to the nucleophile is ahead of bond fission, results in a tight transition state in the upper left quadrant. Full formation of an addition intermediate results in the pentacoordinate intermediate, a phosphorane, shown in the upper left corner.

Once a point of debate, it has been established by stereochemical, LFER, and KIE methods that monoesters undergo reaction by a concerted process. No metaphosphate intermediate is formed except under special circumstances; the best documented is the reaction of *p*-nitrophenyl phosphate in *tert*-butanol as both the solvent and phosphoryl acceptor. [10] Otherwise, the reaction proceeds in concerted fashion with a loose transition state. The evidence and methodology is well reviewed in Lassila et al. [9]. Experimental data indicates that diesters and triesters also generally react via concerted mechanisms, although under some conditions a stable pentacoordinate phosphorane intermediate forms in a stepwise

pathway. Diesters and triesters react with transition states that are successively closer to the upper left of the diagram in Figure 1, with triesters having the tightest transition states.

2.2 Do phosphatases alter the transition state for phosphoryl transfer?

Phosphatase families differ in their catalytic machinery, but a common feature is the presence of cationic residues and/or metal ions at the active site, and a hydrogen bonding network that binds the anionic substrate and stabilizes the transition state. In a tight transition state the charge on the nonbridging oxygen atoms of the phosphate ester substrate increases as the nucleophile attacks. This makes intuitively attractive the notion that a cationic active site should stabilize a tight transition state. Furthermore, to the extent that binding to such an active site neutralizes charge on the nonbridging oxygens, a monoester is made to electrostatically more resemble a diester or triester, and might, therefore, react by the tighter transition states followed by those esters. Triesters are also the most reactive class of phosphate ester, lending credibility to the idea that this might contribute to the catalytic proficiencies of phosphatases. However, the accumulated evidence for PTPs from kinetic isotope effects summarized in this article agrees with a considerable body of experimental evidence,[9] that phosphatases do not alter the transition state for phosphoryl transfer.

2.3 Monoester substrates for KIEs on phosphoryl transfer

We have used the two substrates shown in Figure 2 to measure ^{18}O KIEs on phosphoryl transfer reactions, designed to utilize the remote label method and isotope ratio mass spectrometry to measure KIEs by the competitive method. In our work a nitrogen atom serves as the remote label reporter for the phosphoryl and leaving group O-18 isotope ratios. The essential details of the competitive method, and the remote label method, are summarized here. Both approaches have been described in detail elsewhere.[11]

In the competitive method, a mixture of the light and heavy isotopic isomers is allowed to compete for reaction and the isotope effect is measured from the change in isotopic composition over the course of the reaction. The enzymatic or uncatalyzed reaction in solution is initiated with the desired substrate. The progress of reaction is monitored, and depending on the substrate chosen, the product (either nitrobenzyl alcohol or *p*-nitrophenol) and the unreacted substrate are isolated after varying fractions of reaction, separated, and analyzed using an isotope ratio mass spectrometer (IRMS). If the ^{15}N isotope effect is desired, it is calculated from the IRMS ratios of $^{15}\text{N}/^{14}\text{N}$ in the isolated product and unreacted substrate at known fractions of reaction, which will diverge in different directions from the ratio of isotopes present in the original substrate. One needs the measured fraction f of reaction, and the $^{15}\text{N}/^{14}\text{N}$ ratio of the product (R_p) and that of the remaining starting material (R_s). From the isotope ratio in the original mixture (R_o), the isotope effect can be calculated using equations 1 or 2.[12]

$$\text{isotope effect} = \log(1 - f) / \log((1 - f)(R_s/R_o)) \quad (1)$$

$$\text{isotope effect} = \log(1 - f) / \log(1 - f(R_p/R_o)) \quad (2)$$

The IRMS is very precise, but has the drawback that it measures only small molecule gases that do not undergo fragmentation in the instrument (since such fragmentation will have isotope effects). Thus, one must quantitatively convert the atom of interest into a form the instrument is built to handle, such as N₂, CO, CO₂, or SO₂. Most isotope ratio mass spectrometers incorporate a combustion system that converts nitrogen to N₂ after reduction of the intermediate nitrogen oxides. Thus, if the reactant molecule contains a single nitrogen atom, its isotope ratio is easily analyzed.

The oxygen isotope effects of interest in our work are the ester (bridge) oxygen and the phosphoryl group (nonbridge) atoms. There is no feasible way to quantitatively convert these oxygen atoms in phosphate esters into a molecule such as CO or CO₂ that an IRMS can handle to directly measure oxygen ratios. We have, therefore, used the remote label method, in which the substrate is synthesized in a form such that an isotopic label in the position of mechanistic interest is accompanied by a second isotope label in a position that is easily measured, acting as a reporter. Figure 3 shows *m*NBP as an example of this double-label strategy. To measure the ¹⁸O KIE in the bridge position substrate A was synthesized, with the heavy isotopic isomers of oxygen and nitrogen. This was mixed with isotopic isomer C, which has the light isotope of nitrogen in the reporter position (depleted of even the low natural abundance of ¹⁵N). Because IRMS instruments are most precise when measuring ratios that are close to the natural abundance materials used as calibration standards, the two isotopic isomers A and C are mixed in a ratio close to the natural abundance of nitrogen. When this mixed substrate is used in the competitive experiment described above, the easily measured ¹⁵N/¹⁴N ratio will then function as a reporter for the ¹⁸O/¹⁶O ratios. After correction for incomplete levels of isotopic incorporation in the substrates, and for the ¹⁵N KIE, the ¹⁸O KIE can be obtained.

Figure 3 shows the isotopic isomers that were synthesized and mixed for bridge and nonbridge ¹⁸O KIEs experiments with *m*NBP. The corresponding labeled isomers were used for *p*NPP. No isotopic syntheses is needed to measure the ¹⁵N isotope effects with either substrate, as the natural abundance of nitrogen is already ideally suited for use.

The esters *p*NPP and *m*NBP have leaving groups of different pK_a (7.14 and 14.9, respectively). The classical PTPs hydrolyze phosphotyrosine, which has a leaving group pK_a of ~10. A closely related group of enzymes are the dual-specificity phosphatases, which utilize the same catalytic machinery and mechanism, but have a shallower active site crevice, giving them the ability to hydrolyze phosphorylated serine and threonine residues. The *m*NBP substrate has a leaving group pK_a in the range of the latter substrates. As described below, KIEs obtained with these two substrates have shed considerable light on the details of the chemical mechanism followed by PTPs.

3. KIEs on uncatalyzed phosphoryl transfer of *p*NPP and *m*NBP

The three isotope effects together give a good deal of information about the transition state for hydrolysis of these esters. Two of the KIEs report on the leaving group. The primary isotope effect ¹⁸k_{bridge} gives a measure of the degree of cleavage of the P-O bond. In reactions of *p*NPP, this KIE reaches a maximum value of ~ 1.03 in a late transition state

with nearly full bond fission to the leaving group. The secondary isotope effect ^{15}k measures the negative charge delocalized into the nitro group. The *p*-nitrophenolate anion has contributions from a quinonoid resonance form, and because N-O bonds are stiffer in terms of vibrational frequencies than N-C bonds, the nitrogen atom is more tightly bonded in neutral *p*-nitrophenol than in the phenolate anion. Thus, the ^{15}K EIE in aqueous solution for deprotonation of *p*-nitrophenol is normal, 1.0023 ± 0.0002 . [13] The ^{15}k isotope effect thus gives information as to whether the leaving group departs as the anion, or whether protonation of the leaving group has neutralized all or part of the negative charge resulting from P-O bond fission. This information is important in the application of KIEs measured using this substrate in PTP-catalyzed reactions.

The secondary isotope effect $^{18}k_{\text{nonbridge}}$ reveals whether the phosphoryl group resembles metaphosphate in a loose transition state, or if it is phosphorane-like in an associative mechanism. KIEs are affected both by changes in bond order and changes to bending and torsional vibrational modes. The latter effects can be dominant for secondary isotope effects measured on an atom bonded to a center undergoing a hybridization change. The bond order changes of the nonbridge oxygen atoms in the mechanisms in Figure 1 would lead one to expect $^{18}k_{\text{nonbridge}}$ to be inverse for loose transition states, and normal for tight ones. The bending modes should be in the opposite direction. For example, alpha-secondary deuterium isotope effects are normal for hybridization changes of the type sp^3 to sp^2 or sp^2 to sp . From trends in the data from reactions of phosphate esters (Table 1), bond order changes seem to be the dominant contributors, since $^{18}k_{\text{nonbridge}}$ is normal for diester and triester reactions, and inverse (though very small) for the loose transition states of monoester reactions.

With respect to the leaving group, inferences from KIE data for the uncatalyzed hydrolysis of *p*NPP is consistent with conclusions from LFER experiments, namely, that the transition state is loose and metaphosphate-like. This TS is in the lower-right region of Figure 1, and occurs in a concerted reaction. A true metaphosphate intermediate is ruled out by other data. [9] In contrast, the reaction of *p*NPP in *tert*-butanol (as both solvent and phosphoryl acceptor) was shown by stereochemical results to proceed by a metaphosphate intermediate (and also shows a significantly more positive entropy of activation compared to the aqueous hydrolysis) also must have a very loose transition state. [10, 18] Indeed, the KIE data tell us that, with respect to the leaving group and the phosphoryl group, the TS for the phosphoryl transfer reaction of *p*NPP in water is very similar to the reaction in *tert*-butanol. Most of the difference in the leaving group KIEs in these two reactions can be attributed to the temperature difference, the higher temperature necessary for the aqueous reaction owing to a significantly slower rate.

Depending on pH, a monoester may be present either as the dianion or the monoanion. The monoanion form of *p*NPP (as well as alkyl esters) reacts significantly faster than the dianion. This is attributed to the ability of a proton to transfer from the phosphoryl group (perhaps by the mediation of an intervening water molecule) to the leaving group. This significantly lowers the magnitude of $^{18}k_{\text{bridge}}$ in the monoanion reaction, because loss of the P-O bond (resulting in a normal isotope effect) is partially compensated for by formation of the O-H bond (which results in an inverse effect). The negligible value for ^{15}k indicates the leaving group remains essentially neutral in the transition state of the monoanion hydrolysis, in

contrast to the essentially full negative charge in the transition state of the dianion reaction. The value for $^{18}k_{\text{nonbridge}}$ in the monoanion reaction reflects the known normal isotope effect for deprotonation of a phosphoryl group. [19]

The dianion of the alkyl ester *m*NBP reacts far too slowly to obtain KIE data, so only the monoanion reaction can be studied. In this substrate the nitrogen atom serves only as a remote reporter for ^{18}O , and gives no mechanistic information. As a control, its apparent KIE is always measured and is unity, as expected. An originally reported [16] erroneous inverse bridge $^{18}k_{\text{bridge}}$ KIE for the hydrolysis of *m*NBP monoanion was later corrected[20], and the correct value is 1.0157 ± 0.0009 [17] compared with 1.0094 ± 0.0003 for the hydrolysis of the *p*NPP monoanion (Table 1). The maximum $^{18}k_{\text{bridge}}$ KIE for fission of the P-O bond to the alkyl leaving group in *m*NBP is expected to be about twice that of the aryl leaving group in *p*NPP.[21–23] Thus, the $^{18}k_{\text{bridge}}$ KIEs for both esters are similar in proportion to their respective upper limits, indicating a similar extent of P-O bond fission in their transition states.

The reader will note in Table 1 that the KIEs were obtained at different temperatures for these two substrates. Because temperature affects the magnitudes of isotope effects, it is desirable to measure the KIEs for uncatalyzed reactions in solution at temperatures as close as possible to the corresponding enzymatic reactions being studied. Phosphate monoesters undergo hydrolysis at very slow rates at ambient temperatures, making this impossible for alkyl esters. Even for *p*NPP the monoanion (which reacts faster than the dianion) reacts very slowly under ambient temperatures. We originally measured both the monoanion and dianion reactions at 95°C, where the $^{18}k_{\text{bridge}}$ KIE is 1.0087 ± 0.0003 . [24] For comparisons with enzymatic reactions, an approximation of the temperature effect on the KIEs was made using the equation: $\ln(\text{KIE at } 30^\circ\text{C}) = (368 \text{ K}/303 \text{ K}) \ln(\text{KIE at } 95^\circ\text{C})$. [25] This estimated value of $^{18}k_{\text{bridge}}$ at 30°C of 1.0106 can now be seen as an overestimation of the temperature correction, as when the KIEs for *p*NPP monoanion hydrolysis were later obtained at 30°C [15](Table 1), the experimental value was 1.0094.

The $^{18}k_{\text{nonbridge}}$ KIEs in the reactions of the monoanions of both esters reflect the normal value expected from deprotonation, taking into account the difference in temperatures at which the two reactions were studied. [19]

The KIEs for the uncatalyzed reactions give us important calibration points to compare with data from enzymatic reactions. Knowing the TS for uncatalyzed reactions from the consistent information provided by a variety of physical organic chemistry methods, and having those KIEs in hand, reactions for PTPs can be more confidently analyzed. Advances in computational power have made it feasible to computationally model the hydrolysis of phosphate esters with explicitly included water molecules. Recently, such an approach led to the first good computational approximation of the full set of experimental KIEs on the hydrolysis of *p*NPP, and, consistent with conclusions by experimentalists, found a loose transition state, one that is also similar to that of *p*-nitrophenyl sulfate monoester hydrolysis. [26]

Both the uncatalyzed hydrolysis reactions of the monoanion and dianion have relevance to the reaction catalyzed by PTPs, as well as other phosphatases. Because PTPs take the dianion form of the substrate, the KIE at the nonbridging oxygens can be compared with the uncatalyzed reaction to ascertain whether significant differences exist in the transferring phosphoryl group in enzymatic reactions. The hydrolysis reactions of monoanions share the key feature of protonation of the leaving group in the transition state. The PTPs contain a conserved general acid, an aspartic acid residue, that swings into position to accomplish this task during catalysis. Thus, the basis of comparison for leaving group KIEs in native PTPs is the uncatalyzed monoanion hydrolysis, whereas, if the general acid is removed by mutation, one expects the leaving group to depart as the anion, as in the uncatalyzed dianion hydrolysis.

4. Protein Tyrosine Phosphatases (PTPs)

4.1 PTP mechanism and active site

The classical PTPs hydrolyze only phosphotyrosine residues of polypeptide substrates, while the dual-specific subfamily members (DSPs) also accept phospho-serine and -threonine residues as substrates.[27–30] All PTPs share the conserved signature motif HCXXGXXR(S/T) and a two-step catalytic mechanism with a cysteinyl-phosphate intermediate, shown in Figure 4. The observation of burst kinetics in PTPs indicates that the overall rate-determining step for k_{cat} is hydrolysis of the intermediate.[31–33] The first step is irreversible and phosphoryl transfer from the intermediate occurs only to water.[34] The active sites of all PTP family members are structurally similar and utilize a conserved triad of amino acids in catalysis. These include the nucleophilic cysteine; an arginine, which, along with backbone amides, forms a region called the P-loop that binds substrate and provides transition state stabilization; and an aspartic acid that protonates the leaving group. In the classical, tyrosine-specific PTPs the latter catalytic residue resides on a flexible loop referred to as the WPD loop. The general acid in DSPs is believed to reside on a structurally rigid element.

In PTPs the nucleophile is a thiolate, whereas the KIE model reactions in solution have an oxygen nucleophile (water). Early on in our PTP work we endeavored to find conditions under which KIEs for phosphoryl transfer to a sulfur nucleophile could be measured, but were unsuccessful. Even in neat thiols, the *p*NPP dianion was much less reactive than in water and polyphosphates were produced. Whether these resulted from attack of one *p*NPP dianion on another, or on a fleeting thiophosphate intermediate could not be determined. These failed efforts left a deeper appreciation of how PTPs catalyze phosphoryl transfer to sulfur so effectively, but left us with no experimental information about how the softer sulfur nucleophile might affect the KIEs for phosphoryl transfer. There have been a number of LFER experiments probing the effect of the nucleophile on the transition state, reviewed in [9]. These experiments looked at various oxygen and nitrogen nucleophiles across a range of basicities, and found the nucleophile has a negligible effect on the transition state of phosphoryl transfer reactions from phosphate monoesters.

Table 2 shows the steady-state kinetic parameters for *p*NPP hydrolysis, by the PTPs discussed in this report, for which KIEs have been measured. PTPs are potent enzymes, with

pH optima typically in the range 5.5 to 6. For some PTPs data have been reported that permit a comparison of kinetic parameters with *p*NPP versus phosphopeptide substrates. The substrate has minor effects on k_{cat} , but K_M is significantly smaller, in the micromolar range, for peptide substrates.[35]

4.1.1 Motion during catalysis by PTPs—The WPD-loop has two distinct conformations. In the “open” one the WPD-loop is away from the active site and P-loop. In the catalytically active “closed” conformation, the WPD-loop is folded over the active site, bringing the conserved Asp residue up to 8 Å closer to the bound substrate.[43–47] The loop-open and closed forms of PTP active sites superimpose well with one another except for the WPD-loop itself. This is shown for YopH and PTP1B in Figure 5. The active sites, defined by the P-loops, the conserved arginine, and cysteine nucleophile, align closely in both the ligand-free (loop open) and ligand-bound (loop closed) states. Unlike the WPDloop, there is no evidence for significant motion of the P-loop during catalysis.

Upon oxyanion or substrate binding, the conserved Arg residue rotates to make two hydrogen bonds with the oxyanion. In addition, a new hydrogen bond is formed between the guanidinium group of Arg and the carbonyl oxygen atom of the conserved Trp in the WPD-loop. This assists in stabilizing the WPD-loop in the closed position. This Trp residue is highly conserved in the PTP family, and during WPD loop motion the indole side chain slides in a hydrophobic pocket. As was subsequently found, this residue has interesting and disparate effects in different PTPs, which were diagnosed by kinetics and KIEs.

4.2 KIEs for *p*NPP and mNBP hydrolysis by native PTPs

The isotope effects have been measured for the reaction of *p*NPP catalyzed by the PTP superfamily members YopH from *Yersinia*; PTP1 from mouse; human PTP1B; human VHR; Stp1 from yeast; and human VHZ. [25, 41, 49–51] YopH, PTP1, and PTP1B are classical PTPs with deep active site pockets suited for phosphotyrosine, and also efficiently hydrolyze other phosphorylated phenols. VHZ is an atypical PTP, with the deep active site of classical PTPs but several structural differences, including an immobile loop bearing the general acid.[41, 52] VHR is a dual-specific enzyme (a DSP), while STP1 is Because the KIEs were measured by the competitive method, they are isotope effects on k_{cat}/K_M , (customarily referred to as V/K), which includes the portion of the overall mechanism up to and including the first irreversible step, phosphoryl transfer from the substrate to enzyme in Figure 4.

The isotope effects on *p*NPP hydrolysis are summarized in Table 3. The results for YOP, VHR, PTP1 and PTP1B are very similar and are collected in row 1; the data from Stp and VHZ differ slightly but systematically, and are shown separately. The results from the native enzymes are indicative of a loose transition state in which the phosphoryl group resembles metaphosphate. The KIEs in the leaving group are consistent with extensive bond cleavage, with the leaving group fully neutralized by protonation in the transition state. As in the uncatalyzed hydrolysis of the *p*NPP monoanion, the leaving group is protonated as the P-O bond cleaves, resulting in a near-unity magnitude for $^{15}(V/K)$. In the Stp and VHZ reactions protonation of the leaving group lags behind P-O bond cleavage, evidenced by the small

normal $^{15}(\text{V/K})$ values indicating a partial negative charge on the leaving group, as well as the more normal $^{18}(\text{V/K})_{\text{bridge}}$ KIEs. The $^{18}(\text{V/K})_{\text{nonbridge}}$ KIE results for all of the PTPs are consistent with other data indicating that the active form of the substrate is the dianion. The cumulative data indicate that the transition state of the PTP-catalyzed reaction is not significantly different from that of the uncatalyzed reaction in solution.

The lower part of Table 3 shows KIEs on the reactions of the alkyl ester substrate *m*NBP by YopH [53] and VHR [54]. The bridge isotope effects originally reported may have been subject to the same experimental problem that led to erroneous values originally reported, and later corrected, for the uncatalyzed hydrolysis of this substrate discussed earlier in section 3. Since these $^{18}(\text{V/K})_{\text{bridge}}$ KIEs have not been verified, they are not included in Table 3. The $^{18}(\text{V/K})_{\text{nonbridge}}$ KIEs are sound, and fall within the range found for the aryl substrate and for the uncatalyzed hydrolysis of the *p*NPP dianion. This result implies the phosphoryl groups in the transition states are not significantly different between the alkyl and aryl substrates, and are loose and metaphosphate-like, as in the uncatalyzed hydrolysis in solution. As mentioned earlier, one of the questions in the field was whether substrate binding altered the electrostatic nature of the dianionic monoester to resemble a diester or triester, and thus, also changed the transition state for phosphoryl transfer. The KIEs with native PTPs demonstrate that interactions between the phosphoryl group and the active site do not alter the nature of the transition state from what occurs in uncatalyzed reactions.

4.3 KIEs on catalysis by PTPs having compromised general acids

4.3.1 Mutation of the aspartic acid to asparagine—For both alkyl and aryl substrates, including the activated substrate *p*NPP with its particularly good leaving group, general acid catalysis in PTPs is highly efficient and fully neutralizes the leaving group in the transition state. Mutating the conserved aspartic acid to glutamine causes the expected reductions in rate of several orders of magnitude and loss of the basic limb in pH-rate profiles. KIEs have been measured for the hydrolysis of *p*NPP for the D to N mutants of several PTPs (Table 4) [25, 41, 49–51]. The normal value for $^{15}(\text{V/K})$ demonstrates that the leaving group now departs as the nitrophenolate anion. Loss of the inverse contribution to $^{18}(\text{V/K})_{\text{bridge}}$ leads to a more normal value for this KIE (Table 4). The leaving group now resembles its state in the uncatalyzed hydrolysis of the *p*NPP dianion.

4.3.2 Mutation of the active site arginine—The isotope effects for the R409K mutant of YopH revealed an interesting connection between this conserved arginine residue and the functioning of the general acid. As noted earlier, structural data showed that substrate binding is associated with rotation of the guanidinium group to form bidentate hydrogen bonds with the substrate, as well as formation of a new hydrogen bond with the backbone carbonyl of the conserved tryptophan of the WPD-loop. This reorientation is accompanied by closure of the WPD-loop. The k_{cat} for the R409K mutant of YopH is lower by four orders of magnitude and KIEs resemble those for the general acid mutant D356N (Table 4); thus, the general acid has been rendered nonfunctional.[55] This mutation removes at least one of the two hydrogen bonds provided by arginine in the native enzyme to the phosphoryl group in the transition state. It had long been speculated that such hydrogen bonds might make the transition state for phosphoryl transfer more associative with less bond fission to

the leaving group. The leaving group KIEs for the reaction catalyzed by the R409K mutant are not measurably different from those of the general acid mutants. The rate is an order of magnitude slower than for general acid mutants, demonstrating that removing this hydrogen bond further slows catalysis by adversely affecting transition state stabilization.

4.3.3. Mutations to the conserved tryptophan in the WPD-loop—The Trp residue is highly conserved in the PTP family and is one of the residues in the flexible loop that bears the general acid. While the mobile WPD-loop is found in the classical PTPs, the family members PTP1B and YopH have been used to probe role of the conserved tryptophan in detail (W179 in PTP1B and W354 in YopH). In the superposition of the ligand-free, loop-open structures of these enzymes, the respective indole rings and backbones are adjacent to one another, and the residues adopt very similar positions in the respective loop-closed structures (Figure 5).

The W354F mutation in YopH results in a decrease in k_{cat} by two orders of magnitude (Table 2), loss of the basic limb of the pH-rate profile, and KIEs (Table 4) consistent with the leaving group departing as the anion.[38, 56] These data point to impaired general acid catalysis. Subsequent crystal structures showed that the WPD loop in this mutant is immobile, fixed in a quasi-open position that leaves the Asp 356 side chain too far from the active site to effectively protonate the leaving group.[57] It is interesting to note that the pH-rate profile of this mutant shows that the basic limb is not quite completely abolished; a slight dip is seen in the data above the pH optimum before the levels out.[38] At pH 6 the KIEs for *p*NPP hydrolysis in the leaving group are intermediate between those of native YopH and those of the general acid mutant D356N.[56] The more drastic mutation to alanine in this position (W354A) results in a further reduction in rate, a full flattening of the basic limb of the pH-rate profile, and KIEs that are consistent with total loss of general acid catalysis. The intermediate position of the WPD-loop in the W354F mutant evidently permits an intervening water molecule, bridging the aspartic acid and the substrate, to partially neutralize the leaving group during *p*NPP catalysis. The W354A mutant could not be crystallized, but the kinetic and KIE data suggest that the WPD loop positioning is more compromised than in W354F, and not even partial neutralization of the leaving group is possible. This is testimony to the level of detail that KIEs can reveal about interactions in the transition state that would be very difficult to discern by other means.

Interestingly, the corresponding W179F mutation in PTP1B has no such consequences. Only a minor reduction in k_{cat} of about 2-fold at pH 5.5 results, and the pH-rate profile remains fully bell-shaped.[39] The KIEs (Table 4) are similar to those of the wildtype PTP1B showing that general acid catalysis remains effective. The affinity of the competitive inhibitors tungstate and molybdate for the active site is not affected by the W179F mutation to PTP1B.[39] In contrast, the W354F mutation in YopH reduces the binding affinity for tungstate by about 6-fold.[38] Crystal structures of the W179F mutant of PTP1B show the availability of both the normal loop open and closed positions, consistent with the kinetic results.

These different responses to the conservative Trp to Phe mutation in YopH and PTP1B may be due in part to the opposite direction of motion of the indole side chain during loop

motion. In both enzymes this side chain moves within a hydrophobic pocket, but the residues forming this pocket differ, and the results imply that the pocket in PTP1B exhibits more plasticity than that in YopH. These results suggest that the Trp repositioning upon WPD-loop closure is intimately involved in these dynamic events, affecting catalysis in PTPs.

KIE data from the full body of work with PTPs indicated that all of the enzymes examined carry out catalysis by the same mechanism, with the same transition state, at active sites that crystal structures show are highly superimposable. Yet, turnover numbers vary over several orders of magnitude. The disparate effects on WPD loop function resulting from mutation to the conserved tryptophan in two PTPs suggested that, although the feature of a dynamic WPD loop is conserved in the PTP family, the molecular details involved in the motion of this loop vary. This led us to speculate that catalysis in the PTP family might in fact be tied to different rates of WPD loop motion.

Subsequent NMR studies using YopH and PTP1B in the Loria lab at Yale showed that this is indeed the case. WPD loop motions were measured for both enzymes in the presence and absence of the nonhydrolyzable peptide substrate analog Ac-DADEXLIP-NH₂, where X is difluoromethylphosphono-phenylalanine. Loop dynamic rates were found to differ significantly in the two enzymes, shown in Table 5, and the loop closure rates correlate well with the estimated rates of the first phosphoryl transfer step from substrate to enzyme.[58] This result provided an explanation for the vastly different kinetics of two enzymes that are otherwise chemically and mechanistically indistinguishable, and suggests that modulation of activity within the PTP family as a whole may be modulated by their WPD-loop motion kinetics. In contrast to different loop motion rates, NMR experiments with the phosphate mimic tungstate showed that the rate of diffusion from the active site is the same for YopH and PTP1B, indicating that phosphate release is also the same for the two enzymes, in this final step of the catalytic mechanism.

The slower loop dynamics in PTP1B may reflect its key physiological roles in which turnover rates must meet the requirements of other activities in the cell. In contrast, the YopH enzyme is a virulence factor and the faster loop motions and resulting higher catalytic activity would benefit the disruption of normal cellular processes that facilitate *Yersinia* infection. The intimate knowledge of the chemical mechanism and transition state for PTP catalysis obtained from KIEs provided key information, that led to the discovery that these motions play a crucial role in regulating PTP catalysis.

5. Future directions

Our current work on PTP function and catalysis focuses on learning the reasons for the differences in WPD-loop dynamics between YopH and PTP1B, and, presumably, other PTP family members. We also hope to ascertain whether catalysis is determined primarily by the concentration of the WPD-loop closed form, or, if loop closure is possibly coupled to the chemical step of phosphoryl transfer. As point mutations and loop-swapping experiments proceed, KIEs will be an important part of the toolkit to understand the effect on the

function of general acid catalysis that is intimately connected with protein dynamics in the PTP family.

Acknowledgments

I owe a tremendous debt of gratitude to the late W. W. (Mo) Cleland. Mo was my postdoctoral mentor at UW-Madison, and, later, a family friend who regularly joined us in Logan for the summer opera season. His insights into the potential for the application of KIEs in enzymology helped spawn an area of research that has contributed greatly to the understanding of enzyme mechanisms. I also thank my graduate students and postdocs whose work contributed to the advances described in this article: Rick Hoff, Irina Catrina, Jamie Purcell, Piotr Grzyska, Vyacheslav Kuznetsov, Przemyslaw Czyryca and Tiago Brandao. Our work was supported by NIH grant GM 47297.

Abbreviations

PTP	protein tyrosine phosphatase
DSP	dual specificity phosphatase
KIE	kinetic isotope effect
LFER	linear free energy relationship
IRMS	isotope ratio mass spectrometer
<i>p</i>NPP	<i>para</i> -nitrophenyl phosphate
<i>m</i>NBP	<i>meta</i> -nitrobenzyl phosphate

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Highlights

- Kinetic isotope effects provide mechanistic information about phosphoryl transfer.
- The transition states of enzymatic and uncatalyzed phosphoryl transfer are similar.
- Different PTP catalysis rates are correlated with different rates of protein loop motion.
- Mutations to noncatalytic residues in PTPs affect loop motion and general acid catalysis.

Figure 1. A transition state for phosphoryl transfer in which leaving group bond fission is ahead of bond formation to the phosphoryl acceptor (in this case, water) is referred to as loose, and resides in the lower right region. In the reverse situation, a tight transition state results, in the upper left region. If the sum of the transition state bond orders to the nucleophile and leaving group is unity, the transition state will lie on the synchronicity diagonal.

Figure 2.

The substrates *p*-nitrophenyl phosphate (*p*NPP), left, and *meta*-nitrobenzyl phosphate (*m*NBP), right, showing the nomenclature of bridging and nonbridging oxygen atoms in a phosphate ester, and the positions at which KIEs were measured.

Figure 3.

Isotopic isomers synthesized for measurement of the bridge (A and C) and nonbridge (B and C) ^{18}O isotope effects for *m*-nitrobenzylphosphate using the remote label method. The corresponding isotopic isomers were synthesized for ^{18}O isotope effects measurements with *p*-nitrophenylphosphate. For ^{15}N KIE measurements the natural abundance substrates are used, and no special synthesis of isotopically labeled substrates is necessary.

Figure 4.

The overall mechanism of the PTP-catalyzed reaction. In the first step the phosphoryl group is transferred from substrate to form a phosphocysteine intermediate, which is hydrolyzed in a subsequent step.

Figure 5.

Orientation of key residues at the active site of PTP1B (light blue) and YopH (green). (a) Superimposition of the ligand-free structures with the WPD-loop in the open conformation. (b) Superimposition of the vanadate-bound structures with WPD-loop in the closed conformation, using the same orientation as in (a). The lower, italicized residue positions refer to YopH. For the sake of clarity the only backbone carbonyl group is that of W179/W354 shown in (b). Hydrogen bonds in dotted blue lines are shown only for PTP1B. The PDB IDs used to generate each structure were: PTP1B wildtype: 2CM2 (ligand-free form, open WPD-loop)[48] and 3I80 (VO₄ bound, closed WPD-loop)[49]; YopH wildtype: 1YPT (ligand-free form, open WPD-loop)[47] and 2I42 (VO₄ bound, closed WPD-loop).

Table 1

The range of isotope effects measured for uncatalyzed hydrolysis reactions in solution of phosphate esters with the *p*-nitrophenyl leaving group. The diester and triester KIEs are those for alkaline hydrolysis of esters with the *p*-nitrophenyl leaving group. In this and all tables standard errors in the last decimal place(s) are shown in parentheses. Significant differences in reactivity required the different temperatures at which data were obtained.

Reaction	^{15}k	$^{18}k_{\text{bridge}}$	$^{18}k_{\text{nonbridge}}$
pNPP Dianion, H ₂ O, 95°C	1.0028 (2)	1.0189 (5)	0.9994 (5)
pNPP Dianion, <i>t</i> -butanol, 30°C	1.0039 (3)	1.0202 (8)	0.9997(16)
Diesters	1.0007 – 1.0016	1.0042 – 1.0063	1.0028 – 1.0056
Triesters	1.0007	1.0063 ^a	1.0063 – 1.0250
<i>p</i> NPP Monoanion, 30°C [15]	1.0005 (1)	1.0094 (3)	1.0199 (3)
<i>m</i> NBP monoanion, 115°C [16],[17]	1.0000(1)	1.0157 (9)	1.0151 (2)

^aData from reference [14] Other data is from [11] unless otherwise indicated.

Table 2

Kinetic parameters for hydrolysis of *p*NPP by the PTPs discussed in this report for which KIEs have been measured.

Enzyme	k_{cat} (s ⁻¹)	K_M (mM)	Conditions
YopH ^a	345	2.6	pH 6, 30 °C
YopH D354N ^b	0.89	3.4	pH 6, 30 °C
YopH W354F ^c	2.96	4.8	pH 5.5, 30 °C
PTP1B ^a	34	0.91	pH 6, 30 °C
PTP1B W179F ^d	11.8	0.93	pH 5.5, 23 °C
VHR ^a	6	1.7	pH 6, 30 °C
VHR D92N ^e	0.06	0.42	pH 6, 30 °C
VHZ ^f	3.9	8.3	pH 5.5, 25 °C
Stp1 ^g	3.6	0.08	pH 6, 30 °C
Stp1 D128N ^g	0.009	0.19	pH 6, 30 °C

Literature references:

^a: [36]

^b: [37]

^c: [38]

^d: [39]

^e: [40]

^f: [41]

^g: [42]

Table 3

Kinetic isotope effects for reactions of members of the PTP superfamily with *p*NPP (top, above double line) and *m*NBP (bottom). mNBP: KIEs measured at pH 6.0, 30 C. Standard errors are in the range 0.0001 – 0.0008.

Substrate	Enzyme	$^{15}(V/K)$	$^{18}(V/K)_{\text{bridge}}$	$^{18}(V/K)_{\text{nonbridge}}$
<i>p</i> NPP	YOP, PTP1, VHR, PTP1B	0.9999 – 1.0004	1.0118 – 1.0152	0.9998 – 1.0018
<i>p</i> NPP	Stp1	1.0007	1.0171	1.0007
<i>p</i> NPP	VHZ	1.0013	1.0164	0.9986
<i>m</i> NBP	YopH	0.9996	-	0.9999
<i>m</i> NBP	VHR	1.0000	-	0.9986

Table 4

Kinetic isotope effects for the hydrolysis of *p*NPP by mutants of the PTPs affecting general acid function. KIE data for the general acid D to N mutants of YopH, PTP1, VHR, and Stp1 are very similar to one another, and are therefore shown as a range. For the rest of the data, the numbers in parentheses are the standard errors.

	$^{15}(\text{V/K})$	$^{18}(\text{V/K})_{\text{bridge}}$	$^{18}(\text{V/K})_{\text{nonbridge}}$
D to N mutants: YopH, PTP1, VHR, Stp1	1.0024 – 1.0030	1.0275 – 1.0297	1.0019 – 1.0024
YopH R409K	1.0020 (5)	1.0273 (3)	1.0049 (7)
YopH W354F	1.0013 (2)	1.0240 (10)	1.0015 (8)
YopH W354A	1.0021 (2)	1.0310 (5)	1.0038 (5)
PTP1B W179F	1.0006 (1)	1.0140 (9)	1.0021 (7)

Table 5

Summary of WPD loop motions in YopH and PTP1B at pH 6.5 and 23 C.

Enzyme	$k_{\text{close}} \text{ (s}^{-1}\text{)}$	$k_{\text{open}} \text{ (s}^{-1}\text{)}$	$K_{\text{eq}} \text{ (open/close)}$
PTP1B ligand-free	22 ± 5	890 ± 190	40
PTP1B with peptide bound	30 ± 4	4.5 ± 1	0.15
YopH ligand-free	1240 ± 200	$42,000 \pm 6,000$	34
YopH with peptide bound	1770 ± 240	18 ± 2	0.01