Minireview

Isotope effects in the study of enzymatic phosphoryl transfer reactions

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Abstract Protein-tyrosine phosphatases and serine/threonine protein phosphatases utilize very different catalytic machinery to catalyze phosphoryl transfer reactions. Tyrosine is a better leaving group than serine or threonine, having a pK_a more than three units lower. Has the difference in the catalytic machinery used by these enzyme families evolved as a result of the difference in the lability of their substrates? Are the transition states for phosphoryl transfer similar for the two classes of enzymes? This review summarizes what has been learned from kinetic isotope effects about the nature of enzymatic phosphoryl transfer, and how the enzymatic mechanisms compare to uncatalyzed phosphoryl transfer reactions. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Isotope effect; Phosphoryl transfer; Transition state; Phosphatase

1. Introduction

Because protein phosphorylation plays a central role in regulating cellular processes, protein kinases and phosphatases have been called the Yin and Yang of signaling in the cell [1]. Our research has focused on studies of the details of the chemical mechanism of the phosphoryl transfer reaction catalyzed by phosphatases. These enzymes are of interest not only because of their biomedical relevance, but also their catalytic proficiency. Phosphatases are extremely efficient enzymes, with k_{cat} values ten or more orders of magnitude faster than the rate constants for the uncatalyzed hydrolysis of their normally unreactive phosphate monoester substrates [2,3]. There is considerable interest in whether the enzymatic mechanisms and transition states for phosphoryl transfer differ from the uncatalyzed reactions in solution.

Protein phosphatases (PPase) may be broadly grouped into two families, the protein-tyrosine phosphatase (PTPase) superfamily and the serine/threonine protein phosphatases (Ser/Thr PPase). These enzymes have evolved two very different types of active sites. Perhaps not coincidentally, the leaving groups of their substrates have significant differences. For PTPases, the leaving group is a tyrosine, which has a pK_a of 10. By contrast, the hydroxyl groups of Ser and Thr have pK_a values of about 13.6. The dichotomy is not absolute, as some members of the PTPase superfamily are dual-specific phosphatases and accept both types of substrates. However, it is an intriguing possibility that the different active sites might have evolved in response to this difference in leaving group ability. This minireview summarizes what we have learned about the nature of the phosphoryl transfer process catalyzed by PPases, and how it compares with uncatalyzed phosphoryl transfer in solution.

2. The mechanism of uncatalyzed phosphoryl transfer

Phosphate monoesters, the type of ester processed by phosphatases, typically react via a concerted mechanism with no intermediate [4]. The transition state is characterized by a metaphosphate-like transferring phosphoryl group that has undergone extensive bond cleavage to the leaving group and only a minimal degree of bond formation to the nucleophile (Fig. 1A) (for reviews of the evidence for this mechanism, see [5,6]). Under rare circumstances (in the gas phase, and with the hindered, weakly nucleophilic phosphoryl acceptor *tert*-butanol) [7,8], a free metaphosphate intermediate forms in a two-step reaction. At the opposite end of the mechanistic continuum is the addition–elimination mechanism that involves nucleophilic attack in the first step to form a pentacoordinate phosphorane intermediate. Reactions of phosphate triesters having poor leaving groups follow such a mechanism.

It has been speculated that enzymatic catalysis changes the transition state for phosphoryl transfer involving phosphate monoesters to a more associative one, or perhaps causes a change to a two-step associative mechanism with an intermediate [2,9,10]. Phosphatases utilize different catalytic strategies and functional groups, and comparisons of the structures show that the only similarity is the presence of a positive charge at the active site, in the form of an arginine residue and/or a dinuclear metal center. It is a reasonable suggestion that these metal ions and/or cationic residues might change the normally dissociative phosphoryl transfer process into a more associative one via electron withdrawal from the phosphorus atom, thus promoting nucleophilic attack. Whether or not such a mechanistic change is in fact operative requires direct study.

3. What can be learned from kinetic isotope effects (KIEs)

KIEs can be used to characterize phosphoryl transfer reactions in detail, in particular, yielding information about tran-

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Abbreviations: AP, alkaline phosphatase; KIE, kinetic isotope effect; pNPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; Ser/Thr PPase, serine/threonine protein phosphatase

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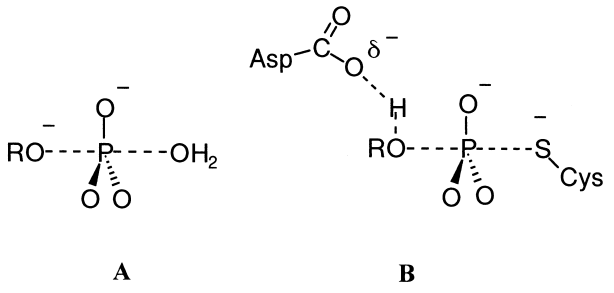

Fig. 1. A: A schematic diagram of the loose transition state for phosphoryl transfer from the dianion of a phosphate monoester to water. The phosphoryl group resembles metaphosphate, the leaving group bears close to a full negative charge, and there is little bond formation with the nucleophile. B: The transition state implied by the KIE data for the reaction catalyzed by PTPases. The dianion is the substrate. The phosphoryl group also resembles metaphosphate, and the leaving group is neutralized by a conserved Asp general acid.

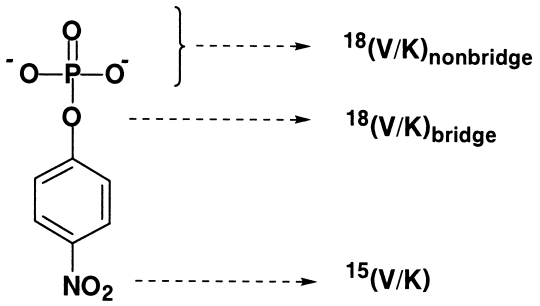

Fig. 2. A diagram of the pNPP substrate showing the positions at which isotope effects are measured. The notation used is that of Northrop [32], where a leading superscript of the heavier isotope is used to indicate the isotope effect on the following kinetic quantity; for example ^{15}k denotes ^{14}k /^{15}k, the nitrogen-15 isotope effect on the rate constant k. The isotope effects are all measured by the competitive method and therefore, the enzymatic isotope effects are effects on k_{cat}/K_M, which includes the portion of the overall mechanism up to and including the first irreversible step.

sition state structure. We have measured the isotope effects for the reactions of *p*-nitrophenyl phosphate (pNPP) in a number of enzymatic reactions, and have compared the results to those for uncatalyzed reactions. Fig. 2 shows the positions at which isotope effects are measured in reactions with pNPP. We use isotope ratio mass spectrometry and the remote label method for measurements of these isotope effects. Our technique requires the presence of a nitrogen atom in the substrate, and this factor, plus the availability of viable synthetic routes, is the reason for our work with this substrate. The methodology used has been described in detail [11,12], as have the ranges in the values of these isotope effects and their use in diagnosing mechanisms and transition states [13].

Table 1 shows the KIEs for the uncatalyzed reactions of the pNPP dianion and monoanion, which give a background for interpreting enzymatic data. In reactions of the dianion, the leaving group departs as an anion in a very late transition state, and the isotope effects in the *p*-nitrophenol leaving group are close to their maximum values. The magnitude of $^{18}k_{bridge}$ reflects the degree of P–O bond cleavage in the transition state, while ^{15}k is a measure of the amount of negative charge developed on the nitrophenyl leaving group. The value for $^{18}k_{non\text{-}bridge}$ is very small and inverse in this loose transition state, in contrast to the phosphorane-like transition states of phosphate diesters and triesters, where this isotope effect is normal and reaches values of 2.5% [13].

In the reaction of the monoanion, a proton is transferred from the phosphoryl group to the leaving group in the transition state. Thus, the magnitude of $^{18}k_{bridge}$ is significantly reduced, because loss of the P–O bond is partially compensated for by formation of the O–H bond. The negligible value for ^{15}k indicates the leaving group remains essentially neutral. The value for $^{18}k_{non\text{-}bridge}$ in this reaction reflects the normal isotope effect for deprotonation of the phosphoryl group [14].

Table 1
KIEs for uncatalyzed phosphoryl transfer reactions of the dianion and monoanion of pNPP

	Dianion	Monoanion
$^{18}k_{non\text{-}bridge}$	−0.03 to −0.07%	1.99 ± 0.03%
$^{18}k_{bridge}$	2.02 to 2.30%	1.06 ± 0.03%
^{15}k	0.32 to 0.39%	0.05 ± 0.01%

The dianion reaction has been examined both in water and in *tert*-butanol, the monoanion reaction in water. Isotope effects are expressed as percent, i.e. 1.0199 = 1.99% and negative values reflect inverse isotope effects, 0.9997 = −0.03%. Standard errors in the data of the dianion reactions are in the range 0.01–0.05%.

4. PTPases

The PTPase superfamily includes two additional groups of enzymes, the VH1-like dual-specificity phosphatases, and the low molecular weight tyrosine phosphatases, which share no discernible sequence identity with the true PTPases [15]. The true PTPases hydrolyze only phosphotyrosine residues of polypeptide substrates, while the dual-specific enzymes process phosphoserine and -threonine residues as well. Despite these differences, these enzymes share virtually identical catalytic sites characterized by a Cys nucleophile, an Arg residue which interacts with the phosphoryl group of the substrate, and an Asp general acid that protonates the leaving group. This Asp residue resides on a flexible loop and lies 8 to 10 Å away from the active site in the resting enzymes, but is brought into position for catalysis by binding of the substrate [16]. The catalytic mechanism of the PTPases proceeds through formation of a phosphocysteine intermediate that undergoes subsequent hydrolysis.

We have measured the isotope effects for the reaction of pNPP catalyzed by the PTPase superfamily members YOP from *Yersinia*, PTP1 from mouse, the dual-specific human VHR, and the low molecular weight Stp1 from yeast [17–21]. Since the isotope effects are measured by the competitive method they are 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. This means that we are probing the phosphoryl transfer from the pNPP substrate to the Cys nucleophile. Results from kinetic studies and the invariance of the isotope effects with pH indicate that the chemical step is rate-limiting in each of these enzymes and thus, the isotope effects reveal actual transition state structure.

These isotope effects are summarized in Table 2. The results for YOP, PTP1 and VHR are very similar and are collected in column 2; the data from Stp differ slightly, but systematically, and are shown in column 3. Column 4 shows the data from the general acid mutants, which are very similar for all four

enzymes. The results from reactions with the native enzymes indicate a transition state that is loose in nature, with minimal nucleophilic participation, and with extensive bond cleavage to the leaving group, which is fully neutralized by protonation in the transition state (Fig. 2). The substrate for all four enzymes is the dianion, and there are no proton transfers involving the phosphoryl group in the reaction. In the Stp reaction, protonation of the leaving group seems to lag a bit behind P–O bond cleavage, and the leaving group bears some negative charge.

In all four of these enzymes, when the general acid Asp is mutated to Asn the values for $^{18}(V/K)_{\text{non-bridge}}$ reveal the transition state has somewhat more nucleophilic participation, although less than that in reactions of phosphodiesters or triesters. The magnitudes of $^{18}(V/K)_{\text{bridge and}}$ $^{15}(V/K)$ indicate extensive bond cleavage to the leaving group, which now bears essentially a full negative charge.

This leads to the conclusion that interactions between the phosphoryl group and the arginine do not fundamentally alter the nature of the transition state of phosphoryl transfer from what is observed in uncatalyzed reactions. In order to further probe the role of this conserved residue, we examined the isotope effects for the R409K and R409A mutants of the PTPase YOP [20]. The data revealed an interesting interplay between the arginine residue and the functioning of the general acid.

It was known from X-ray structural data that when an oxyanion binds to the active site, the Arg-409 residue rotates to form bidentate hydrogen bonds [22]. This reorientation is accompanied by changes in other interactions, resulting in movement of the loop bearing the general acid, bringing this residue into position for catalysis. The values for k_{cat} are lower by about four orders of magnitude for both mutants, but the rate for R409A is about 2.5 times that for R409K. For the reaction catalyzed by R409K, the isotope effects resemble those for the general acid mutant, thus, the general acid is non-functional, and the leaving group departs as an anion. In contrast, in the reaction of R409A, the isotope effects are intermediate between those of the wild type and general acid mutant [20]. This indicates that the general acid is still operative in R409A, although protonation of the leaving group is less complete than in the wild type; the data suggest about 50% charge neutralization. Apparently, a cationic residue in the wrong position (R409K) is more deleterious to the proper movement of the loop bearing the general acid than no cationic residue (R409A) in this position at all. This result emphasizes the danger of interpreting changes in rate to a particular effect without doing a mechanistic analysis. One might have reasonably attributed the lowered catalytic rate to a loss in transition state stabilization afforded by Arg-409, when in fact the majority of the effect is due to a disabling of the general acid.

5. Metallophosphatases

A second large class of phosphatases utilizes a binuclear metal center. The best known member of this group is alkaline phosphatase (AP), which contains two Zn^{2+} ions that play a role in catalysis. The AP reaction proceeds via an intermediate, in which a serine residue (Ser-102 in *Escherichia coli*) is phosphorylated. In this respect, AP differs from the Ser/Thr PPases, which also contain binuclear metal centers, but which catalyze phosphoryl transfer directly to a metal-bound water [23–25].

Our attempts to characterize the transition state of the AP-catalyzed reaction were foiled, as the isotope effects are all near unity [12], consistent with a non-chemical step, such as binding or an associated conformational change being rate-limiting for $k_{\text{cat}}/K_{\text{M}}$. Other evidence for this comes from the viscosity dependence of $k_{\text{cat}}/K_{\text{M}}$ [26].

We found significant isotope effects for the reaction of pNPP by the Ser/Thr PPase calcineurin at its pH optimum, and their magnitudes are significantly larger when reaction occurs at non-optimal pH [27,28]. This indicates that a commitment factor is probably reducing the isotope effects from their intrinsic values. Since it cannot be guaranteed that chemistry is fully rate-limiting even at non-optimal pH, one cannot with assurance draw transition state conclusions from the isotope effect data. It is evident however, that the chemical step is at least partially rate-limiting in this highly regulated enzyme, a point which has been the subject of some contention. The data also indicated that the chemical step of phosphoryl transfer is more rate-limiting with Mg^{2+} than with Mn^{2+}. The data show that the leaving group bears at least a partial negative charge in the transition state with these metal ions, and the non-bridge isotope effects indicate that the transition state lacks a significant degree of nucleophilic participation.

In work with the bacteriophage λ Ser/Thr PPase, we measured significant isotope effects that do not vary when the rate is considerably reduced, whether by varying the pH or by substituting different divalent metal ions (Mn^{2+} or Ca^{2+}) [29]. This is strong evidence for rate-limiting chemistry, and thus, the isotope effects should be the intrinsic ones. The value of $^{18}(V/K)_{\text{non-bridge}}$ is consistent with a transition state having minimal nucleophilic involvement. The leaving group isotope effects indicate that a small amount of negative charge resides on the leaving group in the transition state (Table 2). Ser/Thr PPases have a conserved His residue that has been widely proposed to act as a general acid catalyst, protonating the leaving group [30,31]. The isotope effects for the reaction of the H76N mutant of λ were measured; the isotope effects in the leaving group are indeed elevated in H76N, as expected if the leaving group must bear additional negative charge (Table 2). However, they are significantly smaller than in reactions of PTPases in which the general acid has been mutated. There

Table 2
Isotope effects for reactions of members of the PTPase superfamily and for mutants in which the general acid Asp was mutated to Asn

	YOP, PTP1, VHR	Stp1	D to N mutants: YOP, PTP1, VHR, Stp1	Native λ	H76N mutant of λ
$^{18}(V/K)_{\text{non-bridge}}$	−0.02 to +0.03%	+0.18%	+0.19 to 0.24%	−0.20%	−0.24%
$^{18}(V/K)_{\text{bridge}}$	1.18 to 1.52%	1.71%	2.75 to 2.97%	1.33%	1.83%
^{15}(V/K)	0.00%	0.07%	0.24 to 0.30%	0.07%	0.16%

On the right side of table, the isotope effects for reactions of the Ser/Thr PPase λ and for a mutant in which the putative general acid His was mutated to Asn.

are two possible explanations for this result. It may be that in λ, neutralization of the leaving group is also assisted by coordination to one of the metal ions, or by protonation by a bound water molecule. Alternatively, the smaller leaving group KIEs may simply reflect less transition state bond cleavage to the leaving group.

Perhaps the Ser/Thr PPases utilize a hybrid transition state that is less dissociative with respect to leaving group departure, but that still exhibits the minimal nucleophilic participation typical of the PTPase-catalyzed reactions. Additional work is needed before we can confidently answer this question. In addition, work is underway with substrates having leaving group pK_a values close to that of the natural Ser/Thr substrates, which should shed additional light on these interesting enzymes.

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