The Transition State of the Phosphoryl-Transfer Reaction Catalyzed by the Lambda Ser/Thr Protein Phosphatase

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Received March 2, 1999

Abstract: The catalytic reaction of the Mn^{2+} form of the native bacteriophage λ phosphatase and the H76N mutant was studied with the substrate *p*-nitrophenyl phosphate using heavy atom isotope effects and pH-dependent rate studies. The kinetic isotope effects in the substrate were measured at the nonbridging oxygen atoms [$^{18}(V/K)_{nonbridge}$], at the bridging oxygen atom undergoing bond cleavage [$^{18}(V/K)_{bridge}$], and at the nitrogen atom in the nitrophenol leaving group [$^{15}(V/K)$]. The isotope effects with native enzyme at the pH optimum of 7.8 were 1.0133 ± 0.0006 for $^{18}(V/K)_{bridge}$, 1.0006 ± 0.0003 for $^{15}(V/K)$, and 0.9976 ± 0.0003 for $^{18}(V/K)_{nonbridge}$. These values were constant within experimental error across the pH range from 6.0 to 9.0 and were also unchanged for the slower catalytic reaction resulting when Ca^{2+} was substituted for Mn^{2+}. The results indicate that the chemical step of P−O bond cleavage is rate-limiting, the first metallophosphatase for which this has been shown to be the case. The isotope effects are very similar to those measured for reactions of protein-tyrosine phosphatases, indicating that the two families of enzymes share similar dissociative transition states. The $^{18}(V/K)_{bridge}$ and $^{15}(V/K)$ isotope effects for the H76N mutant were slightly increased in magnitude relative to the native enzyme but were much smaller than the values expected if the leaving group were departing with a full negative charge. The pH vs k_{cat} profile for the native enzyme is bell-shaped with pK_a values of 7.7 ± 0.3 and 8.6 ± 0.4. K_m values for substrate increased with pH approximately 70-fold across the pH range 5.8−9.1. The K_m for the H76N mutant was similar to that observed for native enzyme at high pH and was relatively constant across this pH range. The basic limb of the pH−rate profile is reduced but not abolished in the H76N mutant reaction. The results are discussed in terms of the possible role of His-76 and the nature of the transition state for catalysis in the native enzyme and mutant.

Introduction

The regulation of metabolism in organisms from bacteria to higher eukaryotes is accomplished by the reversible phosphorylation of proteins. Protein phosphorylation (by kinases) or dephosphorylation (by phosphatases) occurs primarily on tyrosine, serine, or threonine residues. Interestingly, phosphatase families have evolved which utilize completely different catalytic machinery to accomplish the hydrolysis of phosphate monoester bonds. The protein-tyrosine phosphatases (PTPases) have no metal ions and utilize a conserved arginine for substrate binding and transition-state stabilization, a cysteine nucleophile to form a phosphoenzyme intermediate, and general acid catalysis is accomplished by a conserved aspartic acid residue which protonates the leaving group.[1−4] Less is known about the mechanistic details of catalysis by the Ser/Thr phosphatases, but these phosphatases are distinguished from the protein-tyrosine phosphatases by their use of a binuclear metal center as a key component of catalysis.[5−7] There are four major Ser/Thr phosphatase families distinguished primarily by substrate specifities and susceptibility to specific inhibitors; these are designated types 1, 2A, 2B (also called calcineurin), and 2C. Among the Ser/Thr phosphatase family crystal structures have been published for the catalytic subunit of rabbit muscle[8] and human[9] PP-1, bovine brain,[10] and human[11] calcineurin, for PP2C,[12] and for the related metalloenzyme purple acid phosphatase (PAP).[13,14] A variety of experimental evidence indicates that the binuclear metal center in bovine brain calcineurin is an Fe−Zn center. The protein phosphatase from bacteriophage λ,

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10.1021/ja990667p CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/25/1999

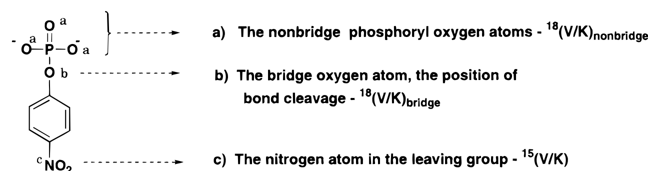

Figure 1. The *p*-nitrophenyl phosphate substrate showing the positions where isotope effects were measured.

designated λ protein phosphatase (λPP), is considered a member of the serine/threonine protein phosphatase family on the basis of sequence comparisons[6] and kinetic and spectroscopic characterizations.[15−17]

The X-ray structures of the members of this family indicate that the binuclear metal center has a ligand environment which is very similar to that of purple acid phosphatases. In PAP the stereochemical course of the reaction occurs with inversion of configuration at phosphorus, supporting a mechanism involving direct transfer of the phosphoryl group to water.[18] The nucleophile has been proposed to be an Fe^{3+}-bound hydroxide ion on the basis of rapid kinetic measurements with anions[19] and pH−rate studies.[20] Kinetic studies, solvent isotope effect data, and redox studies with calcineurin are also indicative of a phosphoryl-transfer mechanism which most likely proceeds by direct transfer to a metal-bound water molecule.[6,21−24]

In addition to the ligands of the binuclear metal center, there are several other conserved amino acids within the region of the active site which could participate in catalysis. One of these is a histidine residue which in calcineurin (H151) is within 5 Å of the two metal ions. A His residue in this region is conserved in other Ser/Thr phosphatases such as PP1 (H125),[6,7] λPP (H76), and purple acid phosphatase.[13,14] It has been proposed that this residue could function as a catalytic general acid in the phosphoryl-transfer reactions catalyzed by this family of enzymes. Mutation of this residue in λPP results in substantial kinetic effects[15,17] and spectroscopic differences.[15]

In this study we report the kinetic isotope effects and pH−rate studies on the reaction of *p*-nitrophenyl phosphate (pNPP) with native λPP and with the H76N mutant. The substrate is shown in Figure 1 with the positions indicated at which isotope effects have been measured. Prior studies of protein-tyrosine phosphatases using this substrate have shown that isotope effects reveal the presence or lack of general acid catalysis in the transition state of the catalytic reaction.[25−27] In the PTPase studies each of the isotope effects revealed alterations in the transition state when protonation of the leaving group in the transition state was lost due to mutation of the general acid.

While a number of PTPases have been mechanistically characterized using isotope effects, similar experiments with phosphatases utilizing binuclear metal ion catalysis have been hindered by the fact that the chemical step is less often rate-limiting with pNPP as the substrate. Isotope effects were measured for the reaction of pNPP with calcineurin.[28] Although calcineurin is very similar to λPP the chemical step of phosphoryl transfer was found to be only partially rate-limiting, leaving the interpretation of the isotope effects in terms of transition-state structure somewhat uncertain.[28,29] Alkaline phosphatase, another phosphatase utilizing binuclear catalysis, was found to have isotope effects of unity, consistent with other data indicating that a nonchemical step is completely rate-limiting for k_{cat}/K_M.[30,31]

In the present study we report strong evidence that the chemical step of phosphoryl transfer is fully rate-limiting for k_{cat}/K_M for the reaction of pNPP with λPP, allowing the full intrinsic isotope effects on the transition state to be observed. In addition the isotope effects for the H76N mutant have been measured, as well as for the native enzyme in which the supplied metal is Ca^{2+} in place of Mn^{2+}, a substitution which results in a reduction in rate of about 17-fold.[16] The results yield information about the transition state of the λPP reaction, the role of His-76, the identity of the substrate as the monoanion or the dianion of pNPP, and the effect of changing the metal ion from Mn^{2+} to Ca^{2+} on the transition state of the reaction. The results also allow an evaluation of the proposal that the normally loose transition state for solution pNPP hydrolysis is altered by coordination of the substrate to the metal ions at the active site and becomes tighter, with greater bond formation to the nucleophile and less advanced bond cleavage to the leaving group. It has been noted that coordination to divalent metal ions in aqueous solution does not alter the transition state of the hydrolysis reaction.[32] However the nature of the transition state for enzymatic phosphoryl-transfer reactions remains controversial.

Experimental Section

Synthesis of Compounds. The bis(cyclohexylammonium) salts of natural abundance *p*-nitrophenyl phosphate, [^{14}N]-*p*-nitrophenyl phosphate, and [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate were synthesized as previously described.[30] [^{14}N]-*p*-nitrophenol and [^{15}N, ^{18}O]-*p*-nitrophenol were synthesized[30] and then mixed to closely reconstitute the 0.365% natural abundance of ^{15}N. This mixture was phosphorylated to produce *p*-nitrophenyl phosphate as the mixture of isotopomers used for the determination of the $^{18}(V/K)_{bridge}$ isotope effect. The [^{14}N]-*p*-nitrophenyl phosphate and [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate isotopic isomers were mixed to reconstitute the natural abundance of ^{15}N, and this mixture was used for measurement of the $^{18}(V/K)_{nonbridge}$ isotope effect. Unlabeled pNPP used for pH versus rate experiments was purchased from Sigma.

Kinetic Isotope Effect Determinations. Isotope effect experiments were run at 100 mM buffer and 1 mM DTT, at 30 °C. The buffers used were MES at pH 6.0 and TRIS at pH 7.8 and 9.0. DTT was omitted at pH 9.0. Reactions were begun with 100 μmol of substrate and

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sufficient λPP used so that background hydrolysis rates were less than 10% of the enzymatic rate. Enzymatic experiments were run in parallel with solution experiments to establish background hydrolysis rates under experimental conditions. The extent of reaction was measured by transferring an aliquot of the reaction mixture into 0.1 N NaOH and assaying for *p*-nitrophenol by measuring the absorbance at 400 nm. Isotope effect experiments were run in triplicate and were stopped at extents of substrate turnover ranging from 40% to 60%. Reactions were stopped by removing the samples from the temperature controller, placing them on ice, and titrating to pH 5. An aliquot was removed for the determination of the precise fraction of reaction. This aliquot was split, with one portion assayed for *p*-nitrophenol immediately and the other portion placed in TRIS buffer at pH 9.0 with alkaline phosphatase for several hours before it was similarly assayed. The ratio of the amount of *p*-nitrophenol in these two samples gave the extent of reaction. The remaining reaction solution was treated to isolate the *p*-nitrophenol product by extracting three times with an equivalent volume of diethyl ether. The aqueous layer, containing the residual substrate, was evaporated briefly under vacuum to remove dissolved ether, an equivalent volume of the TRIS pH 9.0 buffer was added, and the pH was adjusted to 9.0 with NaOH. This sample was treated with alkaline phosphatase to quantitatively hydrolyze all of the remaining substrate. This mixture was then titrated back to pH 5.0 and extracted with ether, the *p*-nitrophenol in this ether fraction representing the residual substrate at the point when the λPP enzymatic reaction was stopped. The ether fractions were dried over magnesium sulfate and filtered, and the solvent was removed by rotary evaporation. The *p*-nitrophenol was sublimed under vacuum at 90 °C, and 1.0−1.5 mg samples were prepared for isotopic analysis using an ANCA-NT combustion system in tandem with a Europa 20−20 isotope ratio mass spectrometer.

Isotope effects were calculated from the isotopic ratio in the *p*-nitrophenol product at partial reaction (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equation 1 was used to calculate the observed isotope effect from R_p and R_o at fraction of reaction *f*.

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

Equation 2 was used to calculate the observed isotope effect from the same reaction using R_s and R_o.[33]

$$\text{isotope effect} = \log(1 - f)/\log[(1 - f)(R_s/R_o)] \qquad (2)$$

R_o was determined from nitrogen isotope ratio mass spectroscopic analysis of the disodium salt of unreacted substrate. As a control this isotope ratio was compared to that from samples of *p*-nitrophenol isolated after complete hydrolysis of the substrate. The agreement of these two numbers demonstrates that, within experimental error, no isotopic fractionation occurs during the isolation and purification of *p*-nitrophenol.

The ^{18}O isotope effects were measured by the remote-label method[34] using the nitrogen atom in the substrate as a reporter for isotopic changes at either the bridging oxygen atom or the nonbridging oxygen atoms, as previously described for solution reactions of pNPP.[30] These experiments yield an observed isotope effect which is the product of the effect due to both the ^{15}N and the ^{18}O substitution. The observed isotope effects from these experiments were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material.[35]

Equilibrium Isotope Effects for Coordination of pNPP to Ca^{+2}. The coordination of pNPP with calcium ion was monitored by recording the shift of the UV−vis spectrum as a function of calcium ion concentration. A 5 mM solution of the disodium salt of pNPP was prepared at pH 7.0 with 100 mM MOPS buffer. Its spectrum was monitored at increasing concentrations of $CaCl_2$ at a constant ionic strength of 3.0 M (NaCl).

An estimation of the nonbridge ^{18}O isotope effect for coordination was performed by monitoring the separation in the ^{31}P NMR signals for ^{16}O- and for ^{18}O-labeled pNPP as a function of the concentration of Ca^{2+}. The solution was 5 mM in pNPP at pH 7.0 (100 mM MOPS buffer). The pNPP consisted of a 1:1 mixture of natural abundance compound and of [nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate. The chemical shifts were followed as calcium ion concentrations were increased from 0 to 1 M at a constant ionic strength of 3.0 M maintained using NaCl. ^{18}O substitution causes a small upfield shift in the ^{31}P chemical shift of about 0.02 ppm/^{18}O. The separation will change as a function of metal ion concentration if there is an isotope effect on the coordination and will reach a maximum at the dissociation constant K_D followed by a decrease as the titration is concluded. This technique was introduced by Ellison and Robinson[36] to determine the equilibrium isotope effect for deprotonation of formic acid and has also been used to measure the isotope effect for deprotonation of phosphate and of phosphate esters.[37]

pH-Dependent Kinetic Assays of λ Protein Phosphatase and λ Protein Phosphatase H76N. The following buffers at 200 mM concentration (2× the final concentration in the assays) were prepared for pH studies: MES for pH 5.8, 6.1, and 6.4; MOPS for pH 6.7, 7.0, and 7.3; Tricine for pH 7.6, 7.9, and 8.2; Bicine for pH 8.5 and 8.8; and CHES for pH 9.1. Stocks of pNPP (0.5 M) were also prepared at each pH by titrating a 1.0 M solution of pNPP with HCl or NaOH to the desired pH followed by dilution with water to the appropriate volume.

Assays contained 100 mM buffer, 1 mM $MnCl_2$, and the appropriate concentration of NaCl to normalize the ionic strength due to varying concentrations of pNPP used in the assays. pNPP was varied in order to determine kinetic parameters at each pH. All reagents except enzyme were incubated for 5 min at 30 °C before initiation of the reaction by addition of enzyme. Reactions were allowed to proceed for 2 min and were terminated by the addition of 1 mL of 2 M TRIS (pH 10) and 10 mM EDTA. At this pH (≥10), *p*-nitrophenol is completely deprotonated. After mixing, the absorbance at 410 nm was read immediately and converted to specific activity using $\epsilon_{410} = 17\,800$ M^{-1} cm^{-1}.

The apparent ionization constants of the enzyme−substrate complex for λPP were determined by fitting the raw data to the appropriate curve using a nonlinear least-squares analysis method.

Separation of pNPP from Contaminant Inorganic Phosphate. A preparation of pNPP containing less contaminating inorganic phosphate was needed in order to carry out assays with λPP and λPP(H76N) at high substrate concentrations to avoid product inhibition (K_i orthophosphate = 0.71 mM at pH 7.8).[16] This was completed by ether extractions of pNPP. A solution of pNPP was prepared and acidified with 1 N HCl to a pH of ∼4.5−5 and extracted twice with ether (ratio of ether to aqueous phase ∼ 3:1). The aqueous phase was retained and acidified to pH ∼1.0 using 1 N HCl. This was then extracted 5−6 times with ether (ratio of ether to aqueous phase ∼3:1). Anhydrous $MgSO_4$ was added to the ether phase as a drying agent and was removed by filtration. The ether was evaporated to dryness using a rotary evaporator, and the yellow oil which remained was dissolved in a few milliliters of H_2O. The pNPP concentration was determined by dilution of the pNPP stock into 0.5 M MOPS, pH 7.0, using $\epsilon_{310} = 9500$ cm^{-1} M^{-1}. The inorganic phosphate concentration of this stock was checked using the Malachite green/ammonium molybdate reagent.[38] Following ether extraction of pNPP, the contamination from inorganic phosphate was 0.14−0.20 mol % compared to about 1.2 mol % in the commercial sample.

Instrumentation. UV−Vis spectra were recorded on a Cary 1 spectrophotometer equipped with a thermostated cell holder. ^{31}P NMR data were obtained using a Bruker ARX400 spectrometer operating at 161.976 MHz. The spectra were the sum of 50−2000 (usually <200) scans and were externally referenced to phosphoric acid (0 ppm) in a coaxial tube. The data were resolution-enhanced by Gaussian apodiza-

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Table 1. Isotope Effects for λPP Reactions with PNPP

enzyme and conditions	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
native, Mn^{2+} (pH 7.8)	1.0006 ± 0.0003	1.0133 ± 0.0006	0.9976 ± 0.0003
native, Mn^{2+} (pH 6.0)	1.0006 ± 0.0002	1.0132 ± 0.0004	0.9981 ± 0.0002
native, Mn^{2+} (pH 9.0)	1.0007 ± 0.0006	1.0143 ± 0.0005	0.9992 ± 0.0001
native, Ca^{2+} (pH 7.8)	1.0007 ± 0.0001	1.0130 ± 0.0004	0.9984 ± 0.0002
H76N, Mn^{2+} (pH 7.8)	1.0016 ± 0.0003	1.0183 ± 0.0009	0.9976 ± 0.0001

tion prior to Fourier transformation. Peak width at half-height was typically 0.018−0.030 ppm.

Results

The isotope effects for the enzymatic reactions of λPP with pNPP obtained from the isotopic ratios of product and those obtained from the isotopic ratios of residual substrate agreed within experimental error in all cases and were averaged to give the results shown in Table 1 with their standard errors.[39] Six or more determinations of each isotope effect were made. Isotope effects were measured with the wild-type enzyme with Mn^{2+} at the pH optimum of 7.8, at pH 6.0 and 9.0, and separately with Ca^{2+} at pH 7.8. Isotope effects with the H76N mutant were measured at pH 7.8 with Mn^{2+}.

The values for the ^{18}O isotope effects have been corrected for the ^{15}N effects and for levels of isotopic incorporation. Since the enzymatic substrate is likely the dianion of pNPP (vide infra), the values for $^{18}(V/K)_{nonbridge}$ in Table 1 at pH 6.0 have been corrected for the equilibrium ^{18}O isotope effect of 1.015 on deprotonation,[37] as previously described.[30] The $^{18}(V/K)_{nonbridge}$ values shown are the isotope effects resulting from ^{18}O in all three nonbridge oxygen atoms. For purposes of comparison the isotope effects for PTPase reactions with the substrate pNPP from previous studies are shown in Table 2.

Data from titration experiments of pNPP with Ca^{2+} are shown in Figures 2 and 3. The UV−vis experiment following the change in λ_{max} of a 5 mM solution of pNPP as a function of Ca^{2+} concentration indicates complete complexation at about 500 mM metal ion; λ_{max} gradually changed from 311.1 nm in the absence of calcium ion to a plateau value of 309.5 at 500 mM Ca^{2+} (Figure 2). The ^{31}P NMR data, however, indicate that further complexation occurs at higher calcium ion concentrations and that saturation is approached but is not complete at 1 M Ca^{2+} (Figure 3). A likely possibility is that the UV−vis experiment detects formation of an initial 1:1 complex, while at higher calcium concentrations a 2:1 metal−pNPP complex forms which causes no further change in the UV−vis spectrum but is detected by changes in the ^{31}P chemical shift. The NMR data indicate that full saturation is approached at approximately twice the Ca^{2+} levels as indicated by the UV−vis experiment. Since full complexation could not be achieved, an exact fit of the data to determine the nonbridge ^{18}O isotope effect for formation of this complex is not possible. An estimation of this isotope effect can be obtained by comparing the maximum change in the isotopic separation induced by Ca^{2+} complexation with that which has been reported for analogous experiments on the protonation of phosphate esters.[40] The magnitude of the isotope effect will be proportional to the maximum *change* in the separation of the peaks of the isotopic isomers. The deprotonation of phosphate monoesters results in a maximum change of 0.015 ppm and an isotope effect of 1.015 ± 0.001 at 27 °C [37] (the isotope effect for protonation will be the reciprocal of this number, 0.985). Since Ca^{2+} results in a maximal change in this separation of 0.0024 ppm, the isotope effect for complexation by two calcium ions is estimated to be about 16% as large as that for protonation of one of the nonbridge oxygen atoms, or approximately 0.997. These $^{18}K_{nonbridge}$ values are the isotope effects resulting from ^{18}O in all three nonbridge oxygen atoms.

Plots of $\log(k_{cat}/K_M)$ vs pH for the native and for the H76N λPP and $\log k_{cat}$ vs pH for the native λPP are presented in Figures4 and 5, respectively. Table 3 contains a listing of kinetic data with pH. Significant substrate inhibition was observed at pH ≤ 7, requiring increased levels of Mn^{2+} in order to obtain saturating concentrations of substrate. For this reason the reaction was not explored below pH 5.8. The theoretical curve through the data in Figure 5 represents the fit to the equation $k_{cat} = k_{cat}\,(\max)/(1 + [H^+]/K_{1app} + K_{2app}/[H^+])$, where K_{1app} and K_{2app} are apparent ionization constants of the enzyme−substrate complex. The value for k_{cat} (max) was 500 ± 200 s^{-1}. K_{1app} and K_{2app} yield pK_a values of 7.7 ± 0.3 and 8.6 ± 0.4, respectively.

Discussion

Factors Influencing the Expression of the Intrinsic Isotope Effects. Experimental data show that the reaction of purple acid phosphatase, which is structurally very similar to the Ser/Thr phosphatases, proceeds with inversion of stereochemistry at phosphorus, consistent with a single-step mechanism.[18] Kinetic and solvent isotope effect data with calcineurin[21,22] also are most consistent with a mechanism involving direct phosphoryl transfer to a metal-bound water molecule without a phosphoenzyme intermediate. This evidence indicates that a model for the catalytic mechanism for the Ser/Thr phosphatases can be represented as shown in Scheme 1. In this scheme k_2 represents a hypothetical conformational change or other nonchemical step after substrate binding. There is no direct evidence for such an additional step in the mechanism of λPP; however it is reasonable to assume for the moment that a conformational change may occur upon substrate binding in order to consider the possibility that a nonchemical step may be partly or fully rate-limiting, thereby affecting the observed enzymatic kinetic isotope effects.

Because the competitive method was used to measure the isotope effects in this study, they are effects on V/K and thus are effects on the part of the mechanism up to the first irreversible step, regardless of which step is rate-limiting in the overall enzymatic mechanism. The first irreversible step is likely to be the phosphoryl-transfer step from substrate to the metal-bound water, which is shown as k_3 in Scheme 1. The justifications for representing this step as irreversible are the poor nucleophilicity of *p*-nitrophenol and the observation that *p*-nitrophenol is a poor inhibitor of Ser/Thr phosphatases,[22] which suggests that its dissociation from the active site is rapid.

When only one step is isotopically sensitive in an enzymatic reaction, the isotope effect on V/K is described by eq 3.[41] In

$$^{*}(V/K) = [^{*}k + c_f + c_r(^{*}K_{eq})]/(1 + c_f + c_r) \qquad (3)$$

this equation $^{*}(V/K)$ represents either $^{18}(V/K)$ or $^{15}(V/K)$, $^{*}k$ similarly designates the isotope effect on the isotope-sensitive

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Table 2. Isotope Effects for PTPase Reactions with PNPP[a]

enzyme and conditions	^{15}(V/K)	^{18}(V/K)$_{bridge}$	^{18}(V/K)$_{nonbridge}$
native PTP1, YOP, and VHR (range)	0.9999 to 1.0001	1.0118 to 1.0152	0.9981 to 1.0003
native Stp1	1.0007 ± 0.0001	1.0160 ± 0.0005	1.0018 ± 0.0003
general acid (D to N) mutants of PTP1, YOP, VHR, and Stp1 (range)	1.0019 to 1.0034	1.0275 to 1.0294	1.0018 to 1.0024

[a] Data from refs 25−27. Data from the native PTP1, YOP, and VHR were very similar and are reported as a range; data from the native Stp1 differ in small but systematic ways from the other three enzymes and are reported separately. Data for all four enzymes are from reactions at their optimum pH. Data from the Asp to Asn general acid mutants of all four enzymes were very similar and are reported as a range.

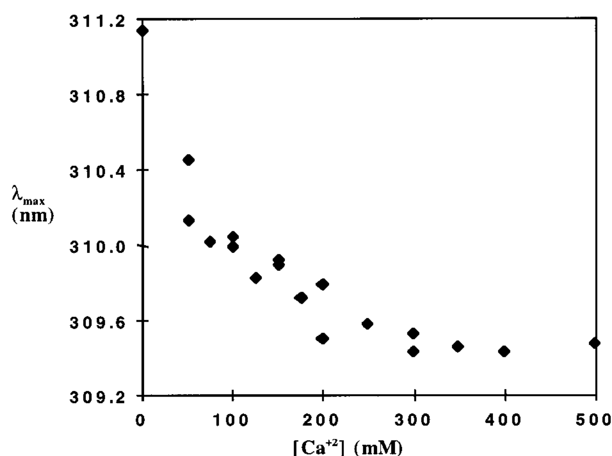

Figure 2. The variation of the λ_{max} of a 5 mM solution of pNPP as a function of calcium ion concentration at pH 7.0, with ionic strength maintained at 3.0 M with NaCl.

step, $^*K_{eq}$ is the equilibrium isotope effect in the forward direction, and the constants c_f and c_r are, respectively, the forward and reverse commitment factors.[42] There will be no reverse commitment if the phosphoryl-transfer step k_3 is irreversible; the data described above indicates that this is the case.[21] With the mechanism in Scheme 1, if the chemical step is the only isotope-sensitive step, then the isotope effect is given by eq 4 and the commitment factor c_f will equal $(k_3/k_{-2})(1 + k_2/k_{-1})$.

$$^*(V/K) = (^*k_3 + c_f)/(1 + c_f) \quad (4)$$

To the extent that a nonchemical step such as substrate binding or a subsequent conformational change is rate-limiting, the resulting forward commitment will suppress the magnitude of the isotope effects on the chemical step and can completely abolish them, resulting in observed isotope effects of unity. This is the case with alkaline phosphatase, where the absence of kinetic isotope effects[30] as well as other kinetic data[31] indicate that a nonchemical step is completely rate-limiting except for substrates having very poor leaving groups. In the calcineurin reaction commitments partially suppress the kinetic isotope effects at the pH optimum, although the suppression is reduced and the isotope effects on the chemical step are fully or nearly fully expressed if the reaction is studied at higher pH.[28]

The ratio k_2/k_{-1} is a measure of the fate of the initial enzyme−substrate complex. If the substrate is tightly bound, this ratio will be large (the substrate will be "sticky") and the isotope effects thereby suppressed. With the λPP enzyme pNPP exhibits a K_M value of 14 mM, a value 30-fold higher than the K_M of a phosphoprotein substrate.[16] This fact gives us a reasonable expectation that the ratio k_2/k_{-1} will be small with pNPP. The ratio k_3/k_{-2} reflects the partitioning of the enzyme−substrate

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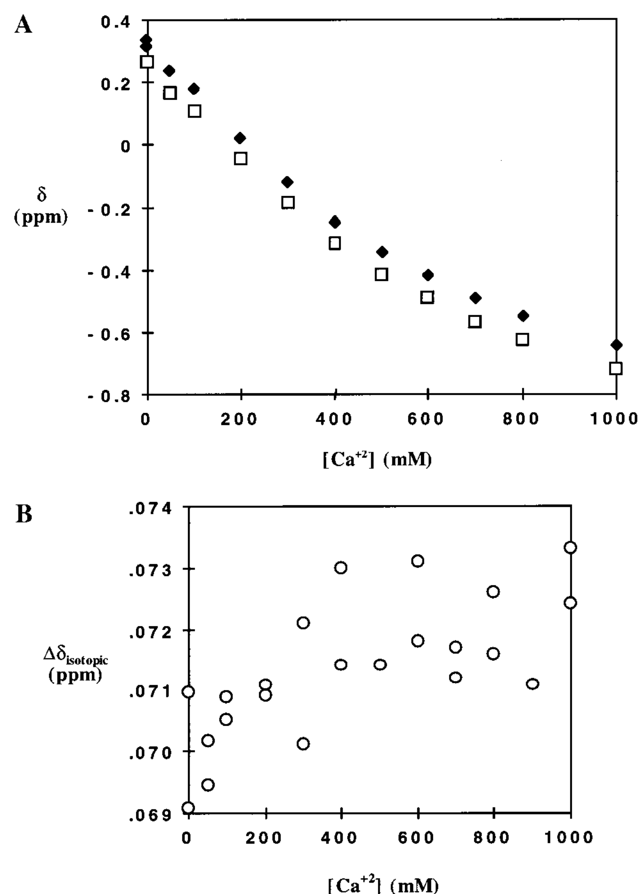


Figure 3. (A) The ^{31}P chemical shift of a 5 mM solution pNPP is shown as a function of calcium ion concentration. Filled diamonds represent the chemical shift of unlabeled pNPP, and open squares represent nonbridge-$^{18}O_3$-labeled pNPP. (B) The difference between the chemical shifts of labeled and unlabeled pNPP is shown as a function of calcium ion concentration. Data from two titrations under identical experimental conditions are shown. For comparison, when the isotope effects for the deprotonation of $^{18}O_3$-labeled phosphate or of $^{18}O_3$-labeled phosphate esters are measured by the same technique, the difference in the isotope-induced chemical shift changes from 0.070 to a maximum of about 0.085 ppm at the pK_a.[40]

complex following the hypothetical conformational change or other nonchemical step. If such an additional step is not rapidly reversible, then the enzyme−substrate complex will partition completely forward from this step and the large k_3/k_{-2} ratio will suppress the isotope effects on the chemical step.

In cases where a commitment factor is sufficiently large to suppress but not entirely abolish isotope effects, the magnitudes of the observed isotope effects will often increase at nonoptimal pH values due to the slower rate of the chemical step and the resultant lowering of the commitment factor, just as was found with calcineurin.[28] As a test for the degree to which chemistry is rate-limiting, the isotope effects for the λPP reaction with pNPP were measured at the pH optimum of 7.8 and also at pH 6.0 and 9.0, where catalysis is significantly slower. The

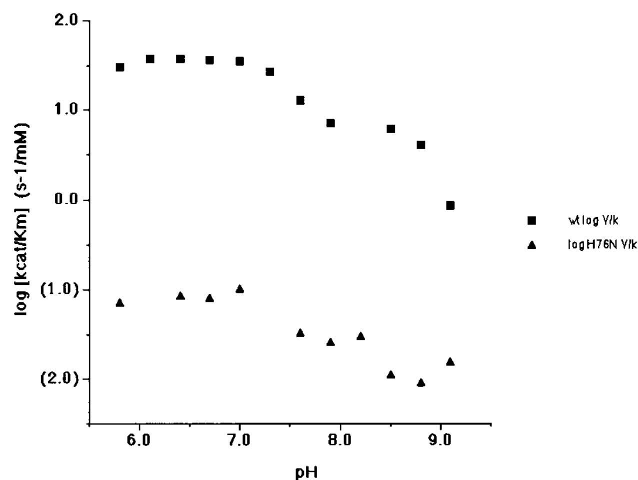


Figure 4. The pH$-k_{cat}/K_m$ profile for the hydrolysis of pNPP by wild type λ protein phosphatase (filled squares) and the H76N mutant (filled triangles).

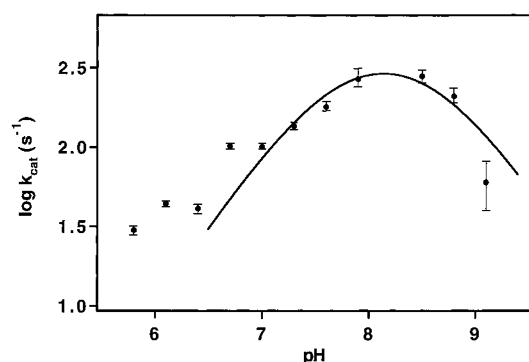


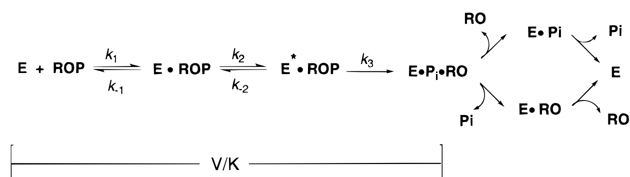
Figure 5. pH$-k_{cat}$ profile for the wild type λ protein phosphatase-catalyzed hydrolysis of pNPP. The line represents the fit to the equation $k_{cat} = k_{cat}(\max)/(1 + [H^+]/K_{1app} + K_{2app}/[H^+])$, where K_{1app} and K_{2app} are the apparent ionization constants of the enzyme$-$substrate complex. K_{1app} and K_{2app} yield pK_a values of 7.7 $\pm$ 0.3 and 8.6 $\pm$ 0.4, respectively.

Table 3. λPP pH$-$Rate Data

pH	k_{cat} (s^{-1})	K_m (mM)	$MnCl_2$ (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Native λPP pH$-$Rate Data				
5.8	30 ± 1	1.0 ± 0.1	15	30
6.1	44 ± 1	1.2 ± 0.1	10	37
6.4	41 ± 2	1.1 ± 0.2	10	37
6.7	102 ± 3	2.8 ± 0.3	10	36
7.0	101 ± 4	2.9 ± 0.5	10	35
7.3	136 ± 4	5.0 ± 0.5	1	27
7.6	180 ±10	14 ± 4	1	13
7.9	270 ± 40	40 ± 10	1	6.8
8.5	280 ± 20	49 ± 7	1	5.7
8.8	210 ± 20	50 ± 10	1	4.2
9.1	60 ± 20	70 ± 30	1	0.86
H76N λPP pH$-$Rate Data				
5.8	1.6 ± 0.1	22 ± 3	15	0.073
6.4	2.2 ± 0.1	26 ± 4	10	0.085
6.7	3.9 ± 0.2	49 ± 5	10	0.080
7.0	4.1 ± 0.5	40 ± 10	10	0.10
7.6	1.0 ± 0.1	30 ± 10	1	0.033
7.9	1.8 ± 0.2	70 ± 10	1	0.026
8.2	0.93 ± 0.08	35 ± 7	1	0.027
8.5	0.57 ± 0.07	50 ± 10	1	0.011
8.8	0.56 ± 0.04	60 ± 10	1	0.0093

magnitudes of the isotope effects are nonunity and, within experimental error, constant over the pH range examined. This strongly suggests that the chemical step is rate-limiting for V/K across the pH range 6.0$-$9.0 and that the isotope effects

Scheme 1



measured are the intrinsic ones for the enzymatic phosphoryl-transfer step with the native enzyme using pNPP as substrate. Since the chemical step is considerably slower in the H76N mutant, and with the native enzyme when Ca^{2+} replaces Mn^{2+}, chemistry should be rate-limiting in those reactions as well.

Compared with the data from the native enzyme, the values of ^{15}(V/K) and of ^{18}(V/K)$_{bridge}$ increase with the H76N mutant. This could indicate a change in the nature of the transition state arising from this mutation. An alternative explanation of the data is that the isotope effects are suppressed in the native enzyme but become fully expressed in the slower H76N mutant. This can be ruled out because a commitment factor in the native enzymatic reaction would suppress all of the isotope effects in equal proportion. Compared to the values for the native reaction, ^{15}(V/K) for the H76N mutant is increased about 2.5-fold while ^{18}(V/K)$_{bridge}$ only increases about 1.3-fold. This means that the changes in the isotope effects caused by the mutation are the result of a change in the transition state for the phosphoryl-transfer reaction. In addition, substitution of Ca^{2+} results in a significant reduction in catalytic rate but no change in the isotope effects which would be expected if the isotope effects were being partially suppressed by commitment factors.

Interpretation of Kinetic Isotope Effects on Phosphoryl-Transfer Reactions. Kinetic isotope effects can characterize reactions in detail, in particular yielding information about the structure of the transition state. The transition state for hydrolysis of phosphate monoesters in solution can be described as very loose or "dissociative" in nature, characterized by extensive bond cleavage to the leaving group and minimal bond formation to the nucleophile, and in which the transferring phosphoryl group resembles a metaphosphate ion.[43,44] Phosphodiesters and triesters exhibit successively tighter, more associative transition states characterized by less bond cleavage to the leaving group and greater bond formation to the nucleophile, where the transferring phosphoryl group resembles a pentacoordinate phosphorane.[43] Linear free-energy relationships indicate that in diesters and triesters with good leaving groups the reactions are concerted with no phosphorane intermediate, but that the transition states become tighter (more associative) than in the dissociative transition state of the monoester reaction.[45,46] Isotope effects have been measured for the phosphoryl-transfer reactions of a number of phosphate esters in solution.[30,35,40,47−49] The cumulative data indicate that isotope effects can distinguish between these types of transition states. Calculations predict inverse

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nonbridge ^{18}O isotope effects for dissociative transition states and normal values for associative transition states.[40] The experimental nonbridge ^{18}O isotope effects for monoester reactions under different conditions are all small and inverse (0.9994−0.9997), and the nonbridge ^{18}O isotope effects for diesters and triesters which have been measured are (with a single exception, which may be anomalous) normal (1.0040−1.0250).

The isotope effects in the leaving group also distinguish between the loose transition states of monoesters and the tighter ones of diesters and triesters. The isotope effects $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ measure charge delocalization in the leaving group and P−O bond cleavage, respectively. The extensive bond cleavage in the monoester dianion reaction shows bridge-^{18}O isotope effects of 1.0202−1.030 and ^{15}N isotope effects of 1.0028−1.0039. The tighter transition states of diesters and triesters with this leaving group exhibit values for the bridge-^{18}O isotope effects in the range 1.0039−1.0060 and for the ^{15}N isotope effect in the range 1.0007−1.0016.

When protonation of the leaving group occurs in the transition state, the normal $^{18}(V/K)_{bridge}$ isotope effect arising from P−O bond cleavage is reduced by the inverse isotope effect arising from protonation. This is just what is observed in the reaction of the pNPP monoanion in solution, where proton transfer from the phosphoryl group to the leaving group occurs during the reaction, and in the reactions of protein-tyrosine phosphatases where protonation of the leaving group is accomplished by a conserved Asp general acid. The maximum $^{18}(V/K)_{bridge}$ effect seen in pNPP reactions in which the leaving group is not protonated is around 1.03, the value observed in mutant PTPases in which general acid catalysis has been eliminated.[25−27] The equilibrium isotope effect for protonation of *p*-nitrophenol is 0.985.[50] Thus when P−O bond cleavage and proton transfer are extensive and synchronous, the observed $^{18}(V/K)_{bridge}$ isotope effect should be close to the product of these values, or about 1.015. This is close to the value measured in reactions of pNPP with PTPases where protonation of the leaving group occurs in the transition state.[25−27]

Mechanism and Transition-State Structure for the λPP Reaction. The bell-shaped pH−rate profile for k_{cat} indicates that maximum activity depends on a species which must be deprotonated and another which must be protonated. The acidic limb most likely represents the nucleophilic metal-bound water molecule. The species responsible for the basic limb results not just in smaller values for k_{cat} but also large increases in K_M when it is deprotonated (Table 3). The K_M values for the H76N mutant at all pH values are more consistent and are similar to those seen in the native enzyme at high pH, suggesting that protonation of this residue assists in substrate binding.

If the substrate for catalysis were the monoanion of pNPP, the rate should increase at low pH as the fraction of the substrate present in solution as the monoanion increases. The pH−rate profile suggests that the catalytically active form of the substrate is the dianion. Due to problems with substrate inhibition below pH 6, the profile could not be extended to low enough pH values where the pK_a of the substrate, which is 4.96 in solution,[51] would be expected to reveal itself. The values for the $^{18}(V/K)_{nonbridge}$ isotope effect give additional evidence that the dianion form of pNPP is the substrate. At pH 6.0 about 10% of the pNPP substrate will be present as the monoanion, whereas at pH 7.8 and 9.0 the concentration of the monoanion will be negligible. Since the isotope effect for protonation of a phosphate monoester is 0.985[37] and if protonation of the substrate occurs before or during catalysis, the observed isotope effect at this position will be the product of the inverse isotope effect for protonation and that for the phosphoryl-transfer step. Since the kinetic $^{18}(V/K)_{nonbridge}$ effects for monoester hydrolysis in all past studies have been very small (near unity), if the monoanionic form is involved then the observed $^{18}(V/K)_{nonbridge}$ effect should be in the neighborhood of the isotope effect for protonation (0.985). The inverse values of $^{18}(V/K)_{nonbridge}$ with λPP were all found to be much smaller (less inverse) than 0.985 (Table 1). Therefore they represent exactly the magnitude expected for a loose transition state of the dianionic substrate, as in the uncatalyzed reaction in solution.

The possibility that coordination to the metal ions at the active site could result in isotope effects at the nonbridging oxygen atoms also must be considered. Metal analysis shows that the purified enzyme is devoid of iron, and no EPR signals for Fe^{3+} are observed. Therefore the active form of the enzyme in these experiments is the di-Mn^{2+} [52] and presumably the di-Ca^{2+} enzyme, depending upon which metal ion is supplied in the buffer solution. A few measurements of ^{18}O isotope effects on the complexation of metal ions to pNPP have been reported. Co(III) is capable of forming inert coordination complexes with phosphate esters such as the Co(ethylenediamine)$_2$−pNPP complex which can be isolated and crystallized. Cleavage of the phosphate oxygen−cobalt bond of this complex results in a kinetic isotope effect of 1.0135,[53] which is close to the equilibrium effect for deprotonation of a phosphate ester. The equilibrium isotope effect for formation of the complex will be the reciprocal of this value, or 0.9866. Formation of the more labile complex between Co(cyclen)$_2$ and pNPP results in a smaller equilibrium effect of 0.9920.[53] Divalent metal ions should exhibit weaker coordination and therefore yield smaller isotope effects. A study of the effects of magnesium complexation with ATP put an upper limit on this ^{18}O isotope effect of 0.999.[54]

We sought to measure, or at least to estimate, the magnitude of the ^{18}O isotope effect for complexation of the pNPP substrate to Ca^{2+}. The log of the stability constant for the Ca^{2+}−pNPP complex has been reported to be 1.26 ± 0.04, as measured by potentiometric pH titration.[55] The determination by UV−vis titration made in this study gave a value of 1.8, which is in reasonable agreement given the less sensitive method of measuring λ_{max}, which changes by only 2 nm from 0 to saturating metal ion. However the results of the ^{31}P NMR experiment indicate that higher-order complexes form as the calcium ion concentration is increased beyond the levels which give 1:1 complexation. The results from the isotope shift ^{31}P NMR experiment (Figure 3) show that there is a very small ^{18}O isotope effect for the coordination of Ca^{2+}. The data allow only an estimation to be made for this isotope effect from a comparison of the maximum separation of the signals of the isotopically labeled ligands compared to the separation observed in the analogous protonation experiment.[37] The change in separation reaches a maximum when the ligand is half protonated (or complexed). The maximum change in the isotopic separation found in the protonation experiment was 0.015 ppm.[37] At a calcium ion concentration which gives half complexation

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of pNPP by the UV−vis data (indicating the 1:1 complex), this change is 0.0014 ppm, suggesting that this isotope effect is at most about 10% as large as for protonation. In the ^{31}P NMR experiments complete saturation of the pNPP ligand could not be achieved and so an exact measurement of the isotope effect for the higher-order Ca^{2+}-pNPP complex could not be calculated, although the isotope effect clearly is greater than for the 1:1 complex. Using the maximum value observed for the change in the isotopic separation gives an estimated value of 0.997, which is probably an upper limit for the isotope effect for the formation of a complex between pNPP and two calcium ions.

The value for the observed $^{18}(V/K)_{nonbridge}$ isotope effect in the λPP reaction will be the product of the isotope effect for binding and that for catalysis. The very small value of the equilibrium isotope effect for coordination of pNPP to two Ca^{2+} ions inferred by the data indicates that binding effects are not likely to be sufficiently large to mask the isotope effect for the phosphoryl-transfer reaction. Thus the $^{18}(V/K)_{nonbridge}$ effect for phosphoryl transfer is essentially unity or slightly inverse, not a normal value that would be indicative of the tighter transition states seen in diesters and triesters. This value is also very close to that observed in the reactions of native PTPases (Table 2), which indicates that the binuclear metal center does not induce a change in the transferring phosphoryl group in the transition state relative to its structure in solution reactions and reactions of PTPases.

The isotope effects in the leaving group for the reaction of the native λPP with both Ca^{2+} and Mn^{2+} are similar to those observed in reactions of the native PTPases, where P−O bond cleavage is extensive in the transition state but the leaving group is neutralized by protonation. The small $^{15}(V/K)$ value of 1.0006 indicates that a small but measurable negative charge develops on the leaving group in the transition state, suggesting that charge neutralization of the leaving group does not quite keep up with P−O bond cleavage. A similar value was previously found for the native low molecular weight PTPase Stp-1 (Table 2).

One of the unanswered questions for the Ser/Thr phosphatases is whether the histidine residue which is conserved in the region of the active site in Ser/Thr phosphatases functions as a general acid. The precise roles of the metal ions are also not known, and it is possible that one or both of them assists in stabilization of the leaving group, analogous to the situation in alkaline phosphatase.[56] Spectroscopic studies have shown that mutation of His 76 results in a perturbation of the ligand environment of the binuclear metal center, possibly by disruption of a hydrogen bond between the histidine and a metal-coordinated water molecule.[15] In the present study mutation of His-76 in λPP to asparagine resulted in an increase in $^{15}(V/K)$ from 1.0006 to 1.0016 and an increase in $^{18}(V/K)_{bridge}$ from 1.0133 to 1.0183 (Table 1). These increases are analogous to those observed in $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ in reactions of PTPases that arise from the mutation of their general acids (Table 2), but the increases in the λPP case are smaller in magnitude. There are two possible explanations for the smaller increase in these isotope effects. One possibility is that the H76N λPP reaction proceeds with an earlier transition state in which P−O bond cleavage is less advanced. An alternative explanation is that charge neutralization of the leaving group is accomplished by one of the metal ions and that the increases in the $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ isotope effects in the H76N mutant are due to perturbation of the ligand environment which interferes with but does not eliminate this interaction in the transition state, rather than the mutation causing the loss of a hypothetical general acid. The $^{15}(V/K)$ isotope effect arises from contributions from a quinonoid resonance structure of the nitrophenolate anion which involves the nitro substituent.[47] If the phenolic oxygen atom is in the region of a cationic metal ion, then less charge will be delocalized into the aromatic ring and this isotope effect will be diminished. This explanation of the data also requires that coordination to the metal ion reduces the $^{18}(V/K)_{bridge}$ isotope effect in the same manner as protonation suppresses the normal isotope effect arising from P−O bond cleavage. There is no experimental basis to allow an estimation of the magnitude of an ^{18}O isotope effect for coordination of nitrophenolate ion to a divalent metal ion. Fairly strong phenolate−metal complexes are known in a number of enzymes, both with ligands (purple acid phosphatase[57]), with substrate analogues (tyrosine hydroxylase[58]), and with substrates (pyrocatechase,[59] Mn^{2+}-dependent catechol dioxygenase[60]). Thus the possibility of a significant isotope effect for such an interaction cannot be dismissed.

The pH−rate profile of the H76N mutant was also determined in an attempt to ascertain the role of this residue. If His-76 functions as a general acid in catalysis, the basic limb of the pH−rate profile for the H76N mutant should be lost. The data for the native enzyme and the H76N mutant are shown in Table 3 and Figure 4. The basic limb which is present in the pH−rate profile of the native enzyme is smaller in the H76N mutant, but is not eliminated. Kinetic data were difficult to obtain due to substrate inhibition at low pH, which required the use of higher metal ion concentrations and increasing K_M values for substrate at higher pH. It is noteworthy that in the native enzyme the K_M increases at high pH to values which are very similar to those found across the entire pH range in the H76N mutant. This implies that protonation of His-76 electrostatically assists in substrate binding (though actual proton transfer to the substrate to form the monoanion is ruled out by the $^{18}(V/K)_{nonbridge}$ isotope effects as discussed earlier). Similar electrostatic assistance in stabilization of the transition state is a reasonable assumption. These facts, and the perturbation of the metal center which results from the H76N mutation, make it difficult to interpret the pH−rate dependency strictly for or against a role for His-76 as a general acid. In sum, neither the isotope effects nor the kinetic data allow a definitive answer to this question. This residue may function more decisively as a general acid in reactions of protein phosphate substrates, where the leaving group is a more basic oxyanion of a serine or threonine residue. If His-76 has a higher pK_a than the *p*-nitrophenolate leaving group, it may merely hydrogen bond to the bridge oxygen atom rather than protonate it.

It is noteworthy that the H76N mutation has no effect on the $^{18}(V/K)_{nonbridge}$ isotope effect. In all of the PTPases it has been found that loss, or even partial interference with the neutralization of the leaving group by the general acid, always resulted in normal $^{18}(V/K)_{nonbridge}$ isotope effects. This was attributed to more nucleophilic participation in the transition state, which can be rationalized by the need for more of a push to expel the leaving group when charge neutralization is lost.[25−27] In the H76N mutant of the λPP the leaving group also departs bearing more of a negative charge, but there is no change in the $^{18}(V/K)_{nonbridge}$ isotope effect. This may be a result of the

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different nucleophile in the λPP reaction (an oxyanion versus a thiolate in the PTPases) or may be due to other electrostatic influences in the catalytic site.

In summary, the isotope effect data are most consistent with a transition state for the λPP reaction which is very similar to those of the PTPase reactions. In the reactions of the native PTPases, the observed $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ isotope effects resulting from bond cleavage and charge development are reduced by protonation of the leaving group. It is conceivable that in the λPP reaction no such neutralization occurs and that the observed $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ isotope effects only serendipitously resemble these isotope effects in the PTPase reactions, and instead reflect an earlier transition state with bond cleavage much less advanced in a tighter transition state, more similar to reactions typical of diesters or triesters. However, if no neutralization of the leaving group occurs in the λPP reaction, it is difficult to explain why the $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ isotope effects increase in the mutant. In addition, phosphoryl-transfer reactions with tighter transition states are characterized by normal $^{18}(V/K)_{nonbridge}$ isotope effects, while the small inverse values for $^{18}(V/K)_{nonbridge}$ seen in this study are characteristic of the loose transition states of monoester reactions. It may also be conceivable that the $^{18}(V/K)_{nonbridge}$ isotope effects for λPP are altered by binding to the binuclear metal center in such a way that they also merely serendipitously resemble the isotope effects seen with monoesters. However the solution results with Ca^{2+} and the fact that the H76N mutation, which is known to perturb the metal center, alters the isotope effects in the leaving group but *does not change* the $^{18}(V/K)_{nonbridge}$ isotope effect, suggest that this isotope effect is not significantly altered by substrate binding. Thus the simplest explanation of the data is that the transition state for the λPP reaction is similar to those of the reactions of PTPases.

Conclusions

It has been suggested that the presence of positive charge in phosphatases around the transferring phosphoryl group implies a transition state with associative character and considerable nucleophilic participation[61,62] in contrast to the reaction in solution which is dissociative or loose in nature. The data obtained in this study do not lend support to the notion that the binuclear center in the λPP induces such a mechanistic change. The isotope effects are most consistent with a reaction similar to the reaction in solution and in the reactions of native PTPases, which is noteworthy in light of the vastly different catalytic machinery utilized by the two classes of phosphatases. The substitution of Ca^{2+} for Mn^{2+} in the binuclear metal center also does not result in a change in the transition state for phosphoryl transfer. The H76N mutation results in small changes in the isotope effects, indicative of greater negative charge borne on the leaving group in the transition state. Neither the isotope effect data nor the pH−rate data clearly answer the question of whether His-76 functions as a general acid in the enzymatic reaction, and it is still uncertain whether this residue or the metal ions participate in charge neutralization of the leaving group. The kinetic data indicate that substrate binding is assisted when His-76 is protonated.

Acknowledgment. Financial support of this work came from NIH Grant GM47297 to A.C.H. and Grant GM46865 to F.R. and from the US Army Advanced Civil Schooling Program for support of R.H.H. R.H.H. and P.M. contributed equally to this work.

Supporting Information Available: Table of pNPP λ_{max} data as a function of calcium ion concentration shown graphically in Figure 2; table of the ^{31}P chemical shift data shown in Figure 3; and a sample ^{31}P spectrum from the calcium ion complexation experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

JA990667P

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