Transition-State Structures for the Native Dual-Specific Phosphatase VHR and D92N and S131A Mutants. Contributions to the Driving Force for Catalysis[†]

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ABSTRACT: Isotope effects have been measured for the reaction of the human dual-specific phosphatase VHR with *p*-nitrophenyl phosphate (pNPP). Isotope effects in the nonbridge oxygen atoms, in the bridge oxygen atom, and in the nitrogen atom were measured by the competitive method using an isotope ratio mass spectrometer. These are isotope effects on *V*/*K*, and give information on the chemical step of phosphoryl transfer from substrate to the enzymatic nucleophile Cys-124. With native VHR, $^{18}(V/K)_{\text{nonbridge}} = 1.0003 \pm 0.0003$, $^{18}(V/K)_{\text{bridge}} = 1.0118 \pm 0.0020$, and $^{15}(V/K) = 0.9999 \pm 0.0004$. The values are similar to the intrinsic isotope effects for the uncatalyzed reaction, indicating that the chemical step is rate-limiting with the pNPP substrate. The transition-state structure resembles that for the uncatalyzed reaction and those previously found for the protein-tyrosine phosphatases YOP51 and PTP1, and is highly dissociative with P−O bond cleavage and protonation of the leaving group by the general acid Asp-92 both well advanced. The D92N mutant exhibits a transition state similar to that of the uncatalyzed reaction of the pNPP dianion, dissociative and with the leaving group departing as the nitrophenolate anion. The S131A mutation causes an increase in the pK_a of the nucleophilic Cys, but the isotope effect data are unchanged from those for the native enzyme, indicating no effects of this increase in nucleophilicity on transition-state structure. The double mutant D92N/S131A manifests both the increase in pK_a of the nucleophilic Cys and the loss of general acid assistance to the leaving group. In the absence of the general acid, the change in nucleophile pK_a results in an increase in $^{18}(V/K)_{\text{nonbridge}}$ from 1.0019 (with D92N) to 1.0031(with D92N/S131A), indicating loss of P−O nonbridge bond order in the transition state. It is concluded that this is more likely caused by electrostatic effects rather than resulting from increased nucleophile−phosphorus bonding in a less dissociative transition state, although the latter explanation cannot be excluded on the basis of the present data. Electrostatic effects between the thiolate anion nucleophile and the phosphoryl group may be an important part of the driving force for catalysis in this family of enzymes.

Tyrosine and threonine/serine phosphorylation of specific proteins is a necessary event in the transmission of information from the surface of the cell to the nucleus through signal transduction pathways which mediate cell growth and differentiation. In signaling pathways, the interplay of protein kinases and protein phosphatases provides the proper attenuation of the signal. Protein phosphatases having the active-site motif HCxxGxxRS(T) are involved in a wide variety of cellular responses (Stone & Dixon, 1995), and are generally grouped into two classes: protein-tyrosine phosphatases (PTPases)[1] and dual-specific phosphatases. Within each class, the amino acid sequence identity is approximately 30%; however, between these classes, the sequence identity is much less (5%) and appears to be limited to catalytically important residues. Although the dual-specific phosphatases are capable of dephosphorylating both phosphotyrosine and phosphoserine/threonine residues, several reports have suggested that the PTPases and the dual-specific phosphatases use a common mechanism of phosphate monoester hydrolysis. The human phosphatase VHR (***V***accinia **H**1-**r**elated) is the archetypal enzyme in studies designed to elucidate the catalytic mechanism for the entire class of dual-specific phosphatases. The conserved Cys-124 is the catalytic nucleophile and forms a thiophosphate intermediate (Zhou et al., 1994; Denu et al., 1996). Evidence suggests that VHR employs an aspartic acid (Asp-92) as a general acid to facilitate catalysis (Denu et al., 1995a). Site-directed mutagenesis and kinetic studies also indicate that a conserved hydroxyl side chain from S(T) is critical for efficient hydrolysis of the phosphoenzyme intermediate (Denu & Dixon, 1995). Similar mechanistic studies on the PTPases indicate that these enzymes also proceed through a thiophosphate intermediate (Cho et al., 1992) and use a conserved aspartic acid as a general acid (Zhang et al., 1994a; Denu et al., 1996). Collectively, these findings suggest that despite the lack of sequence identity, the PTPases and the dual-specific phosphatases may utilize a common mechanism of

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[1] Abbreviations: pNPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; VHR, vaccinia-H1-related; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino[tris(hydroxymethyl)methane; DTT, dithiothreitol; D, aspartic acid; N, asparagine; C, cysteine; S, serine; A, alanine. The notation used to express isotope effects is than of Northrop (1977) where a leading superscript of the heavier isotope is used to indicate the isotope effect on the following kinetic quantity; for example, $^{15}k_3$ denotes $^{14}k_3/^{15}k_3$, the nitrogen-15 isotope effect on the rate constant k_3.

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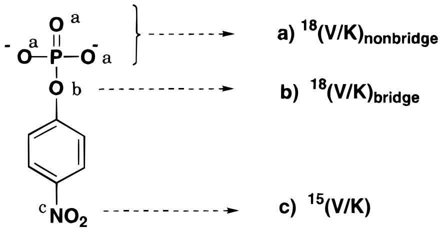


FIGURE 1: pNPP substrate showing the positions where isotope effects were measured in the enzymatic reactions (on *V*/*K*) and the solution hydrolysis reactions (on *k*).

catalysis. The experiments described in the current report were designed to test this hypothesis and compare the transition state for phosphoryl transfer in VHR with that recently described for two PTPases (Hengge et al., 1995a). In addition, the catalytic contribution of conserved Asp-92 and hydroxyl (Ser-131) residues was probed.

A considerable amount of evidence indicates that phosphate monoesters in aqueous solution hydrolyze by a concerted S_N2 (A_ND_N in the IUPAC nomenclature) mechanism involving a dissociative metaphosphate-like transition state, where cleavage of the bond to the leaving group is nearly complete and bond formation to the nucleophile is very small (Bourne & Williams, 1984; Skoog & Jencks, 1984; Herschlag & Jencks, 1989). Kinetic isotope effect measurements are also consistent with a dissociative transition state for the nonenzymatic hydrolysis of phosphate monoesters (Gorenstein et al., 1977; Hengge et al., 1994). However, it has been proposed that enzymatic phosphoryl transfer reactions may prefer an associative mechanism since catalytic groups could stabilize the resulting increased negative charge in the phosphoryl group in the transition state (Knowles, 1980; Hasset et al., 1982). Recently the enzymatic phosphoryl transfer reaction from phosphate monoester substrates to a Cys nucleophile in an alkaline phosphatase mutant has been proposed to proceed via an associative transition state with less bond cleavage to the leaving group (Han & Coleman, 1995).

A detailed description of the transition-state structures involved in these reactions can be obtained from kinetic isotope effects. In a phosphoryl transfer reaction, the secondary O-18 isotope effect in the nonbridge oxygen atoms reveals whether the transferring phosphoryl group in the transition state is metaphosphate-like as expected in a dissociative mechanism, or if it resembles a pentavalent phosphorane as expected in an associative mechanism. The primary O-18 isotope effect in the leaving group, at the site of P−O bond cleavage, gives an indication of the degree of cleavage of this bond in the transition state. When *p*-nitrophenyl phosphate is the substrate, the N-15 isotope effect in the leaving group is sensitive to the amount of negative charge delocalized into the aromatic ring, thus giving a measure of the charge developed on the leaving group in the transition state (Hengge & Cleland, 1990). The positions of these three sites of isotope effect measurement in the substrate are shown graphically in Figure 1. All three of these isotope effects have previously been measured for the well-characterized solution hydrolysis reactions of the pNPP monoanion and dianion (Hengge et al., 1994) as well as for two members of the PTPase family, the *Yersinia* enzyme and rat PTP1 (Hengge et al., 1995a). These data provide a background to interpret the isotope effects obtained in this study on the reactions with the native dual-specific VHR phosphatase and with the mutants D92N, S131A, and the double mutant D92N/S131A.

In this report, we also consider the source of the catalytic power utilized by the enzyme in the phosphoryl transfer step from the substrate to the enzymatic Cys nucleophile.

MATERIALS AND METHODS

Materials. Alkaline phosphatase from *Escherichia coli*, type III, and natural-abundance pNPP were purchased from Sigma. Diethyl ether was distilled before use. [^{14}N]-*p*-Nitrophenol, [^{14}N]-*p*-nitrophenyl phosphate, [^{15}N,^{18}O]-*p*-nitrophenol, and [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate were synthesized as previously described (Hengge et al., 1994).

Site-directed mutagenesis and construction of expression vectors have been described for the D92N (Denu et al., 1995a), S131A (Denu & Dixon, 1995), and double D92N/S131A (Denu et al., 1996) VHR mutant enzymes. Overexpression of protein was performed using a pT7 vector transformed into BL21/DE3 bacteria. All proteins were purified to homogeneity in the identical manner previously described for native VHR (Denu et al., 1995b). Mutant enzymes displayed the same chromatographic properties as native enzyme when purified across a cation exchange column (S-Sepharose) and a size exclusion column (G-75 Sephadex), suggesting that the mutations did not affect the overall structure. To avoid possible contamination from previous VHR preparations, each mutant was purified across a new S-Sepharose column.

Isotope Effect Measurements. Isotope effect experiments run at pH 6.0, 7.0, and 8.0 were performed in buffer solutions consisting of a mixture of 0.1 M Bis-Tris and 0.1 M Tris, with 2 mM DTT. All experiments were run at 35 °C. In each experiment, approximately 100 μmol of the appropriate isotopic mixture of *p*-nitrophenyl phosphate was dissolved in 10 mL of buffer. After the solutions were equilibrated at 35 °C, sufficient enzyme (native VHR, D92N, or S131A) was added so that half the substrate was hydrolyzed after about 2 h. Reactions were run in triplicate and were allowed to progress for varying times to obtain fractions of reaction varying from approximately 20 to 70%. With the slower double mutant D92N/S131A, sufficient enzyme was used to give a half-life for reaction of about 48 h. A control experiment under identical conditions but without enzyme showed that uncatalyzed hydrolysis of substrate was negligible ($<$0.5%) after 72 h. The progress of the reactions was monitored by following production of *p*-nitrophenol from the absorbance at 400 nm of an aliquot of the reaction mixture added to 0.2 N NaOH. Reactions were stopped by chilling in an ice bath; after a 50 μL aliquot was removed for purposes of determining the fraction of reaction (see below), the solutions were titrated to pH 5 with HCl. The *p*-nitrophenol was extracted with diethyl ether (3 × 25 mL). The ether extracts were dried over magnesium sulfate and concentrated to dryness by rotary evaporation.

The aqueous layer after the ether extractions contained the unreacted *p*-nitrophenyl phosphate substrate. After residual ether was removed from the solutions by brief rotary evaporation, they were brought to pH 9, treated with 1 unit of alkaline phosphatase, and allowed to stand overnight to completely hydrolyze the remaining pNPP. The *p*-nitrophe-

Table 1: Kinetic Isotope Effects on Reactions of VHR and Mutants with pNPP

	VHR Reactions[a]		
pH	$^{15}(V/K)$	$^{18}(V/K)_{\mathrm{bridge}}$	$^{18}(V/K)_{\mathrm{nonbridge}}$[b]
native (pH 6)	0.9999 ± 0.0004	1.0118 ± 0.0020	1.0003 ± 0.0003
native (pH 7)		1.0113 ± 0.0019	
native (pH 8)		1.0130 ± 0.0012	
D92N (pH 7)	1.0030 ± 0.0002	1.0294 ± 0.0009	1.0019 ± 0.0005
S131A (pH 7)	1.0002 ± 0.0003	1.0119 ± 0.0005	1.0001 ± 0.0006
D92N/S131A (pH 7)	1.0029 ± 0.0003	1.0306 ± 0.0010	1.0031 ± 0.0007
	Solution Reactions[c]		
	^{15}k	$^{18}k_{\mathrm{bridge}}$	$^{18}k_{\mathrm{nonbridge}}$
monoanion	1.0005 ± 0.0002	1.0106 ± 0.0003	1.0224 ± 0.0005
dianion in water	1.0034 ± 0.0002	1.0230 ± 0.0005	0.9993 ± 0.0007
dianion in *tert*-butanol	1.0039 ± 0.0003	1.0202 ± 0.0008	0.9997 ± 0.0016

[a] Reaction conditions: 0.1 M Bis-Tris/Tris, 2 mM DTT, 10 mM substrate, 35 °C. See Materials and Methods for additional details. [b] The isotope effect due to ^{18}O in all three nonbridge oxygen atoms. [c] Data from Hengge et al. (1994).

nol so produced was isolated by acidification to pH 5 and ether extraction.

To measure the fraction of reaction at the point of stoppage, the 50 μL aliquot described above was added to 1 mL of Tris buffer, pH 9, containing 1 mM Zn and 1 mM Mg. The solution was assayed for *p*-nitrophenol, treated with alkaline phosphatase, allowed to stand overnight to completely hydrolyze the remaining substrate, and then assayed again.

All *p*-nitrophenol samples were further purified by sublimation under vacuum at 90 °C and then combusted to produce nitrogen gas, which was analyzed by isotope ratio mass spectrometry as previously described (Hengge et al., 1994).

The isotope effects were calculated from the isotopic ratios of nitrogen in the substrate (R_{o}), in the product *p*-nitrophenol after partial reaction (R_{p}), and in the residual substrate after partial reaction (R_{s}) using eqs 1 and 2, where f is the fraction of reaction. The independent calculation of each isotope effect using R_{o} and R_{p} and using R_{o} and R_{s} from eqs 1 and 2, respectively, provides an internal check of the results. The isotopic ratio R_{o} was determined in two ways: from nitrogen obtained from combustion of samples of the pNPP substrate, and separately by combustion of *p*-nitrophenol obtained after complete hydrolysis of pNPP with alkaline phosphatase and workup as described above. The value of R_{o} obtained by the two methods was identical within experimental error, demonstrating that the procedures used to recover and purify *p*-nitrophenol do not result in isotopic fractionation which could interfere with isotopic effect measurements.

$$\text{isotope effect} = \log (1 - f)/\log [1 - f(R_{\mathrm{p}}/R_{\mathrm{o}})] \quad (1)$$

$$\text{isotope effect} = \log (1 - f)/\log [(1 - f)(R_{\mathrm{s}}/R_{\mathrm{o}})] \quad (2)$$

The ^{18}O isotope effects were measured using the remote-label technique (O'Leary & Marlier, 1979) using multiply labeled ^{15}N, ^{18}O pNPP as previously described (Hengge et al., 1994). Experiments with these substrates yield an isotope effect which is the product of the ^{15}N and the ^{18}O isotope effects. The observed isotope effects obtained from these experiments were corrected (Caldwell et al., 1991) for incomplete levels of isotopic incorporation in the substrate and for the ^{15}N isotope effect which was separately determined using natural-abundance pNPP.

Medium Effects on Catalysis. Enzymatic assays with pNPP as substrate were performed as described (Denu et al., 1995b). Organic solvents (methanol, ethanol, glycerol, ethylene glycol, DMF, and DMSO) were added up to a concentration of 5 M. In each case, the change in ionic strength of the buffer was corrected for by the addition of the appropriate amount of water.

Rapid-Reaction Kinetics. Rapid-reaction kinetics with the double mutant D92N/S131A were carried out as described (Denu & Dixon, 1995). The rate of intermediate formation as a function of pH value was determined and the analyzed as previously described (Denu et al., 1995a).

RESULTS

The isotope effects for the enzymatic reactions of VHR with pNPP are given in Table 1 together with their standard errors. The isotope effects 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 together to give the results in the table. Six or more determinations of each isotope effect were made. With the wild-type enzyme, isotope effects were measured at the pH optimum of 6.0, and the value for $^{18}(V/K)_{\mathrm{bridge}}$ was also measured at higher pH values where the rate of the chemical step is slower (Denu et al., 1995a). Isotope effects with the mutant proteins were determined at pH 7.

The values for the ^{18}O isotope effects have been corrected for the ^{15}N effects and for levels of isotopic incorporations. Since the enzymatic substrate is the dianion of pNPP (Denu et al., 1995a), the values for $^{18}(V/K)_{\mathrm{nonbridge}}$ in Table 1 have also been corrected for the equilibrium ^{18}O isotope effect on protonation, as previously described (Hengge et al., 1994). The $^{18}(V/K)_{\mathrm{nonbridge}}$ values are the isotope effects resulting from ^{18}O in all three nonbridge oxygen atoms.

All of the isotope effects in this study were measured by the competitive method. Therefore, they are isotope effects on V/K, and are insensitive to all steps after the first irreversible step. For purposes of comparison, the isotope effects for the solution hydrolysis reactions of pNPP from a previous study are included in Table 1. The aqueous hydrolysis reactions were performed at 95 °C, and the values in Table 1 have been corrected to 35 °C using the equation ln (IE at 35 °C) = (368 K/308 K) ln (IE at 95 °C).

The effects of various organic solvents on the kinetics of pNPP hydrolysis by VHR and the mutants S131A and D92N were determined. In all cases, the rate of enzyme-catalyzed hydrolysis was significantly higher in organic solvent than in 100% water. For native VHR at pH 7, the ratio of $k_{cat}/k_{cat(5\ M\ methanol)}$ was 0.57 ± 0.01, and the $(V/K)/(V/K)_{5\ M\ methanol}$ was 0.22 ± 0.01. At pH 6, the k_{cat} effect was 0.29 ± 0.02. Similarly, the k_{cat} effects (pH 7) for the S131A and D92N mutants were 0.59 ± 0.01 and 0.80 ± 0.01 in 5 M methanol, respectively. To determine whether this medium effect was specific to methanol, other solvents were tested. These experiments yielded similar results. In 5 M ethanol (pH 7, S131A VHR), glycerol, ethylene glycol, DMF, and DMSO, the k_{cat} effects were 0.77 ± 0.01, 0.79 ± 0.01, 0.82 ± 0.01, 0.72 ± 0.01, and 0.82 ± 0.01, respectively.

Stopped-flow experiments with the double mutant D92N/S131A revealed that the rate of intermediate formation followed the lone ionization from the thiol of Cys-124. The apparent pK_a value was 6.69 ± 0.15, and the pH-independent rate was 0.66 ± 0.10 s^{-1}. The pK_a value of the cysteine in the native enzyme was previously shown to be 5.5 (Denu et al., 1995a).

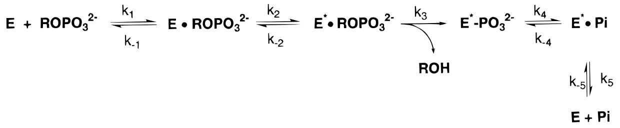
DISCUSSION

The isotope effects observed for the nonenzymatic solution hydrolysis reactions of pNPP are the intrinsic values for the chemical step of phosphoryl transfer from this substrate to an acceptor, since the commitments which often suppress isotope effects in enzymatic reactions are not present in the uncatalyzed reactions. The isotope effects on the aqueous hydrolysis reactions of the dianion and monoanion of pNPP, and on the reaction of the dianion of pNPP in *tert*-butanol, are given in Table 1. The aqueous reactions are thought to be concerted in nature, with bond formation to the nucleophile and P−O bond cleavage occurring in the same step. The transition state is dissociative, with the P−O bond to the leaving group largely broken and with minimal bond formation to the nucleophile. The phosphoryl transfer in *tert*-butanol is thought to occur stepwise with a discrete metaphosphate intermediate (Friedman et al., 1988). To the extent that the substrate is desolvated in the active site of the VHR enzyme (Yuvaniyama et al., 1996), the reaction environment may more nearly resemble *tert*-butanol than water. As the isotope effect data show, the transition states for the reactions of the dianion of pNPP in water and in *tert*-butanol are not significantly different and are consistent with nearly complete breakage of the bond to the leaving group (Hengge et al., 1994).

In these reactions, the leaving group departs as the *p*-nitrophenolate anion. By contrast, in the monoanion hydrolysis, the leaving group is protonated as it departs. Thus, in this reaction, the observed ${}^{18}k_{bridge}$ isotope effect is a product of the normal effect for bond cleavage and of the inverse effect for protonation, and the resulting value is smaller than that observed in the dianion reactions. Thus, the isotope effects ${}^{18}k_{bridge}$ and ${}^{15}k$ for monoanion hydrolysis in solution represent expected values for nearly complete bond cleavage where the leaving group is protonated in the transition state.

Previous measurements of the isotope effects in the nonbridge oxygens of phosphate monoesters, diesters, and triesters yield values that are negligible or slightly inverse for dissociative mechanisms typical of monoesters, values that are small but normal (usually from 0.28 to 0.56%) for diesters of *p*-nitrophenol (Hengge et al., 1995b) where the transition state is somewhat associative, and values that are larger and normal (0.60−2.50%) for triesters (Caldwell et al., 1991) where the reaction is more associative.

Scheme 1



The substrate for the VHR phosphatase, as for all PTPases known to date, is the dianion of the phosphate monoester (Denu et al., 1995a). Kinetic studies indicate that the leaving group is protonated by the general acid Asp-92; thus, the leaving group isotope effects ${}^{18}(V/K)_{bridge}$ and ${}^{15}(V/K)$ in the enzymatic reactions should be compared with the values of ${}^{18}k_{bridge}$ and ${}^{15}k$ from the uncatalyzed monoanion reaction because in both reactions the leaving group is protonated in the transition state. The nonbridge oxygen isotope effects in the enzymatic reactions should be compared with those for the dianion reaction because no proton transfer involving these atoms occurs. The magnitudes of the leaving group isotope effects should be large for the chemical step of bond cleavage, but will be negligible for binding of pNPP or for a conformational change. If unfavorable partitioning of intermediates occurs, the resulting commitment factor will diminish these observed isotope effects relative to those expressed in the solution reaction.

The PTPases cleave phosphorylated substrates by a two-step mechanism via a phosphoenzyme intermediate, depicted in Scheme 1. In the native VHR enzyme, Asp-92 is believed to serve as a general acid catalyst in the first chemical step, which is transfer of the phosphoryl group to the nucleophilic Cys-124 (Denu et al., 1995a). The rate-determining step in the overall mechanism with the native enzyme is formation of the phospho-enzyme intermediate. Mutation of Ser-131 to alanine results in a change in the rate-limiting step to the hydrolysis of this intermediate (Denu & Dixon, 1995). 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 only the first 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 in likely to be the phosphoryl transfer step from substrate to the active site nucleophile Cys-124, 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 PTPases (Zhang et al., 1994b) 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 (Cleland, 1987). In this equation, $*(V/K)$ represents either ${}^{18}(V/K)$ or ${}^{15}(V/K)$, $*k$ similarly designates the isotope effect on the isotope-sensitive 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 (Northrop, 1977). There will be no reverse commitment if the phosphoryl transfer step k_3 is irreversible, and if it is the only isotope-sensitive step, then the expression for the isotope effect reduces to eq 4. The commitment factor c_f will equal $(k_3/k_{-2})(1 + k_2/k_{-1})$.

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

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

It is unusual for the chemical step to be completely rate-limiting in the reaction of an enzyme with its natural substrate. To the extent that nonchemical steps are rate-limiting, the resulting forward commitment will suppress the magnitude of the isotope effects on the chemical step, or completely abolish them (resulting in observed isotope effects of unity). 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 and the isotope effects thereby suppressed. With the dual-specific phosphatase VHR, the alternate substrate pNPP exhibits K_m values which are an order of magnitude higher than those for physiologically relevant phosphotyrosine peptides (Denu et al., 1995b). 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 complex following a possible conformational change bringing the peptide loop bearing the catalytic general acid near the active site. It is not yet certain whether the VHR enzyme shares this feature, but this has been a characteristic of other protein phosphatases in this family (Schubert et al., 1995; Jia et al., 1995). This conformational change occurs upon binding of substrate, and if it 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.

The $^{18}(V/K)_{\mathrm{bridge}}$ effect is the largest of the isotope effects measured, and is therefore the most sensitive test for the presence of commitments which may be diminishing the observed isotope effects from their intrinsic values on the chemical step. The magnitude of $^{18}(V/K)_{\mathrm{bridge}}$ was measured at the pH optimum of 6.0, and also at pH 7.0 and 8.0 (where k_{cat}/K_m is reduced by 2 orders of magnitude) as another test for the presence of a commitment. 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, as the rate of the chemical step is slower and the commitment factor therefore smaller. The magnitudes of $^{18}(V/K)_{\mathrm{bridge}}$ are large at the pH optimum of 6.0 and are similar to the value for the uncatalyzed solution reaction, and within experimental error are unchanged over the pH range tested. This strongly suggests that k_3 is the rate-limiting step for V/K at the pH optimum, and that the isotope effects measured are the intrinsic ones for the enzymatic phosphoryl-transfer step with the native VHR enzyme and the pNPP substrate. Since the chemical step in the mutant enzymes is considerably slower than in the native VHR, chemistry can be assumed to be rate-limiting in those reactions as well.

An alternative explanation of the data in Table 1, namely, that the isotope effects are suppressed in the native enzyme but become fully expressed in the slower D92N mutant, can be ruled out by two considerations. First, the D92N mutation reduces k_{cat}/K_m by about the same amount as does a change in pH from the optimum of 6 to 8 with the native enzyme (Denu et al., 1995a). Thus, both changes should reduce any commitment factors by the same degree. The values of $^{18}(V/K)_{\mathrm{bridge}}$ with the native enzyme at pH 6 and at pH 8 are the same within experimental error. This indicates that the larger value observed with the D92N mutant results from changes in transition-state structure due to the loss of general acid catalysis, rather than from a change in the commitment factor. Second, a significant commitment factor in reactions with the native enzyme would suppress all of the isotope effects by the same proportion, which is not observed in these experiments. Compared to the values for the D92N mutant, $^{18}(V/K)_{\mathrm{bridge}}$ for the native enzyme is lower by 60% while $^{15}(V/K)$ and $^{18}(V/K)_{\mathrm{nonbridge}}$ are completely abolished.

Transition-State Structures. (A) Native VHR. The results with native VHR indicate a highly dissociative transition state very similar to the uncatalyzed solution reaction. The $^{18}(V/K)_{\mathrm{nonbridge}}$ effect indicates that the central phosphoryl group is metaphosphate-like in the transition state, similar to its structure in the dianion reaction in solution. The $^{15}(V/K)$ effect of unity indicates that proton transfer to the leaving group has fully compensated for P−O bond cleavage, making the leaving group neutral in the transition state. This differs from what was found in the solution reaction of the monoanion, where proton transfer from a nonbridge oxygen to the leaving group seems to be slightly behind P−O bond cleavage, as indicated by a small ^{15}k isotope effect (Hengge et al., 1994). The value for $^{18}(V/K)_{\mathrm{bridge}}$ indicates that bond cleavage to the leaving group is far advanced.

While the assumption that the chemical step k_3 is the only isotope-sensitive step is valid for the isotope effects in the leaving group, isotope effects on binding are possible for the nonbridge oxygen atoms. In the enzyme−substrate complex, these oxygens are near an arginine residue which is believed to assist in substrate binding and/or transition-state stabilization. If there is an isotope effect on binding of substrate, then the observed value of $^{18}(V/K)_{\mathrm{nonbridge}}$ will be the product of the equilibrium effect on binding and the kinetic effect on the phosphoryl transfer step. Such a binding effect on the nonbridge oxygens due to hydrogen bonding or partial protonation will be inverse. Protonation of a phosphate esters has an inverse oxygen-18 isotope effect of about 1.6% (Knight et al., 1986). The fact that the observed $^{18}(V/K)_{\mathrm{nonbridge}}$ value in the native VHR reaction is not significantly different from its value in the uncatalyzed reaction of the dianion in solution is evidence that substrate binding is not isotope-sensitive. This indicates that the interaction between the active site arginine and the nonbridge phosphoryl oxygen atoms is electrostatic and does not involve proton transfer, which would result in significant inverse isotope effects (Knight et al., 1986).

(B) The D92N Mutant. Aspartate 92 is believed from other studies to serve as the general acid which protonates the leaving group in the native VHR reaction, and replacement of this residue with asparagine results in the loss of the basic limb from the pH−rate profile and a decrease of 100-fold in rate (Denu et al., 1995a). Consistent with loss of transition-state protonation of the leaving group, the isotope effects $^{18}(V/K)_{\mathrm{bridge}}$ and $^{15}(V/K)$ reveal that in the D92N mutant reaction the leaving group departs as the *p*-nitrophenolate anion with considerable negative charge delocalized into the aromatic ring and with cleavage of the P−O bond to the leaving group nearly complete, similar to the uncatalyzed reaction of the dianion in solution. Interestingly, unlike the solution reaction, the value for $^{18}(V/K)_{\mathrm{nonbridge}}$ is slightly normal with this mutant. A similar effect was observed with general acid mutants of two PTPases (Hengge et al., 1995).

This indicates decreased nonbridge P−O bond order in the transferring phosphoryl group in the transition state, compared to the reaction of the native VHR or the solution dianion reactions.

(C) The S131A Mutant and the D92N/S131A Double Mutant. The loss of the conserved hydroxyl group of S131 in VHR results in a decrease in k_{cat} of 100-fold, while k_{cat}/K_m values are reduced only 2−4-fold (Denu & Dixon, 1995). As a result, the rate-limiting step in catalysis by the S131A mutant is hydrolysis of the phosphoenzyme intermediate. It has been proposed that the hydroxyl of Ser-131 assists in hydrolysis of the intermediate by hydrogen bonding to the sulfur atom of Cys-124, which is the leaving group in this step of the mechanism. Kinetic analysis and titration experiments indicate a small increase in the pK_a of the Cys-124 thiol by about 0.3 unit in the S131A mutant (Denu & Dixon, 1995b). In the double mutant, rapid-reaction kinetics suggest that the apparent pK_a of the thiol has been shifted to 6.7, more than 1 pH unit above its value in the native enzyme. Since the isotope effects in this study are effects on V/K, they allow us to examine the effects of the S131A mutation on the chemistry and transition-state structure of the first phosphoryl transfer step even though subsequent phosphoenzyme hydrolysis is the rate-limiting step of the overall mechanism.

Within experimental error, all three isotope effects with the S131A mutant VHR (Table 1) are the same as with the native enzyme, indicating that the increased pK_a of the nucleophilic Cys has no detectable consequences in transition-state structure. In the double mutant S131A/D92N, which lacks general acid assistance to departure of the leaving group, the increase in the pK_a of the Cys nucleophile does have a measurable effect on the transition state of the phosphoryl-transfer step. The leaving group isotope effects $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ are unchanged from those in the D92N mutant, indicating similar degrees of bond cleavage to the leaving group and negative charge development on the leaving group. The $^{18}(V/K)_{nonbridge}$ isotope effect, however, is significantly larger (more normal) than for the D92N mutant. The transferring phosphoryl group in the transition state is even less classically metaphosphate-like in regard to nonbridge P−O bond order than in the reaction of the general acid mutant. This change in the transition state with the double mutant is not accompanied by any change in leaving group bond cleavage, and in this respect, it is still clearly dissociative. The decrease in nonbridge P−O bond order could in theory either be a result of direct nucleophilic participation (S−P bond formation in the transition state) or may be electrostatic in origin. These possibilities are considered separately.

Nucleophilic Participation. The isotope effect data obtained in reactions with the native VHR indicate that the transition state strongly resembles that for the uncatalyzed solution reaction, both in terms of exhibiting advanced bond cleavage to the leaving group and in terms of the metaphosphate-like structure of the transferring phosphoryl group. Although none of the isotope effects in this study directly measure nucleophile−phosphorus bond formation, a change to a more associative transition state with significant nucleophilic participation should produce accompanying consequences in the other bonds affected in the reaction, and this is not observed.

In the reactions with the two mutants lacking general acid assistance to the leaving group, the data indicate some decrease in bond order between the phosphorus atom and the nonbridge oxygens, an effect more pronounced in the double mutant, while bond cleavage to the leaving group is still far advanced in both reactions. Especially with the double mutant, the value for $^{18}(V/K)_{nonbridge}$ is similar to values observed in reactions of activated phosphodiesters where *p*-nitrophenol is the leaving group (Hengge et al., 1995b). These latter reactions are concerted, but in contrast to the dissociative transition states typical of monoesters, these active diester reactions have transition-state structures with significant bond formation to the nucleophile and much less bond cleavage to the leaving group. However, the isotope effects with the VHR mutants show that leaving group bond cleavage remains far advanced, symptomatic of the dissociative transition state with minimal nucleophilic bond formation which is typical of monoester reactions. Another indication that the $^{18}(V/K)_{nonbridge}$ effects may not arise from nucleophile−phosphoryl bond formation in the transition state is the greater loss of P−O bond order indicated in the reaction of the double mutant bearing the higher pK_a nucleophile. If nucleophilic participation is significant, the Hammond postulate predicts that the stronger nucleophile of the double mutant should exhibit an earlier transition state, with less advanced nucleophile−phosphorus bonding and accompanying smaller loss of P−O nonbridge bond order, and probably less advanced bond cleavage to the leaving group as well. This is the opposite of what is observed in the nonbridge P−O bond order, and leaving group bond cleavage is unaltered.

Electrostatic Interactions. An alternative explanation for the origin of the bonding changes in the phosphoryl group in the transition state lies in the consideration of the net charge in the transition states of the reactions studied, and in the resonance structures for metaphosphate ion. Figure 3 shows a cartoon of the transition states, and their respective net charges, for the reactions of pNPP as the dianion in solution, with native VHR, and with the VHR mutant D92N. In each reaction, the transition state resembles an extended trigonal bipyramid, with the phosphoryl group's nonbridge oxygen atoms in the equatorial plane and the apical positions occupied by the nucleophile and leaving group. In the solution reaction, the nucleophile (water) is neutral. In the dissociative transition state, the leaving group has been nearly completely expelled as the anion, leaving the phosphoryl group with a single negative charge, giving a net charge of −2. In the transition state of the enzymatic reaction with native VHR, the nucleophile has a negative charge, the phosphoryl group has a single negative charge, and the departing leaving group has been neutralized by the general acid, giving an overall net charge of −2. In the general acid mutants, the leaving group departs as the anion, resulting in a transition state having a net charge of −3. Computational studies indicate that metaphosphate is more accurately portrayed as a resonance hybrid of the structures shown as opposed to the traditional representation A (Rajca et al., 1987; Horn & Alrichs, 1990). In the reactions of the VHR mutants lacking general acid catalysis, the additional negative charge in the transition state should enhance the contributions of resonance forms of the metaphosphate-like phosphoryl group with its charge dispersed as much as possible, which are structures B and C in Figure 2. A resulting decrease in

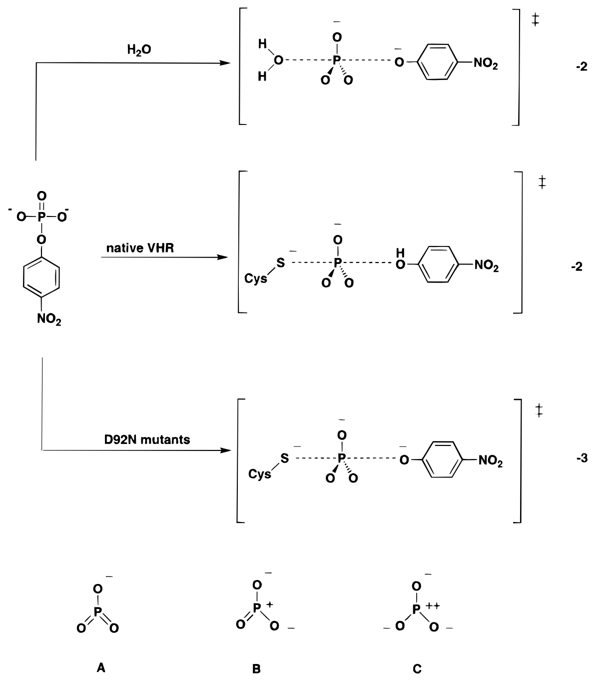


FIGURE 2: Transition-state structures of the reactions of pNPP dianion with water, with native VHR, and with general acid mutants of VHR, showing the overall charge on the transition state. At the bottom are the resonance structures contributing to the structure of the metaphosphate ion. The transferring phosphoryl group in the transition states of these reactions resembles the metaphosphate ion.

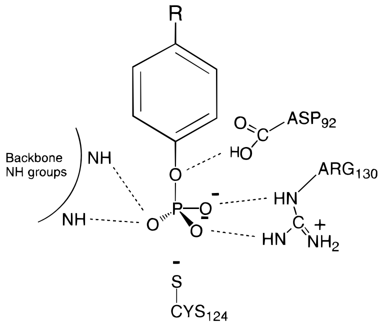


FIGURE 3: Enzyme−substrate complex formation. Binding and positioning of the phosphate group of the substrate involve a conserved Arg-130 and backbone amide NH groups (Yuvaniyama et al., 1996). The arginine forms a bidentate interaction with two of the equatorial nonbridge oxygen atoms. The third equatorial oxygen is positioned by hydrogen bond interactions with the amide NH groups of the conserved active site loop HCXXGXXRS. The nucleophilic thiolate anion is found at the base of the active site cavity and in a position to facilitate P−O bond cleavage by charge repulsion.

P−O bond order is consistent with the isotope effect results. Analogous bond order effects in phosphates as a result of electrostatic interactions resulting from ion pairing have been reported (Baraniak & Frey, 1988). In the native VHR and the D92N mutant, hydrogen bonding with the Cys thiolate anion lowers its pK_a and disperses the negative charge. The stronger negative charge of the higher pK_a Cys anion in the double mutant may result in a greater delocalization of negative charge in the phosphoryl group in the transition state, rather than in greater S−P bond formation. This explanation for the origin of the lessened nonbridge P−O bond order in the general acid mutants is more consistent not only with the effect of nucleophile pK_a on this transition-state bond order but also with the precedent of enzymatic monoester phosphoryl transfers being dissociative in nature with little nucleophilic bond formation, like their solution counterparts (Hollfelder & Herschlag, 1995). One exception which has been reported is the alkaline phosphatase mutant in which the nucleophilic Ser-102 is replaced by Cys, where evidence was presented indicating increased nucleophilic participation in an associative mechanism with decreased bond cleavage to the leaving group (Han & Coleman, 1995). In all of the enzymatic reactions in this study, bond cleavage to the leaving group remains nearly complete regardless of the mutation. This suggests that nucleophilic participation in the form of S−P bond formation remains very small in each case.

The Role of the General Acid. It has recently been proposed that the driving force for the phosphorylation step in the VHR reaction (and phosphatases in general) is activation of the substrate by means of a preequilibrium protonation of the bridge oxygen atom by the general acid Asp-92, or by an enforced hydrogen bonding interaction between these atoms (Zhang et al., 1995). The observation of an inverse solvent isotope effect of 0.52 in the enzymatic phosphorylation reaction with the substrate pNPP was cited as evidence for preequilibrium protonation.

Solvent isotope effects on enzymatic reactions must always be interpreted with considerable caution due to medium effects, the multiple enzymatic isotopic exchanges that occur upon changing from H_2O to D_2O, and other considerations (Quinn & Sutton, 1991). In particular, inverse solvent isotope effects can be the result of the difference in viscosity between D_2O and H_2O (Karsten et al., 1995). With VHR, apparent inverse "isotope effects" are observed upon the addition of viscosigens or organic solvents. Both native and mutant VHR enzymes displayed an inverse effect of between 0.5 and 0.8 when the reaction solution was made 5 M in either glycerol, ethylene glycol, DMSO, DMF, methanol, or ethanol. In view of these results, the possibility that the reported inverse solvent isotope effect is due to a medium effect cannot be ruled out.

The pK_a of $C_6H_5OH_2^+$ is −6.7 (Arnett & Wu, 1960). The pK_a of the bridge oxygen in phenyl phosphate should not be much different, since the pK_a of the phosphoramidate monoanion $H_3N^+{-}PO_3^{2-}$ is close to that of NH_4^+ (Chanley & Feageson, 1963). If the effect of the nitro group is similar to that in nitrophenol, the pK_a of the bridge-protonated pNPP anion will be −9.7, and probably somewhat lower (Kirby & Varvoglis, 1967). In contrast, in the late transition state, this pK_a will be close to that of free *p*-nitrophenol, or 7.1. The general acid Asp-92 has a pK_a of 7.2 in the enzyme−substrate complex (Denu et al., 1995a). Thus, proton transfer to the leaving group will be far more favorable in the transition state rather than in the ground state to form a bridge-protonated substrate.

Although it is an important contributor, protonation of the leaving group is not the major driving force for the enzymatic reaction. Comparison of the uncatalyzed aqueous hydrolysis rates of pNPP (Kirby & Varvoglis, 1967) with the VHR-catalyzed rates (Denu et al., 1995a) reveals that VHR accelerates the reaction of the pNPP dianion substrate by (7×10^6)-fold. The D92N mutant, which has lost the general acid catalyst, still exhibits an acceleration of (6×10^4)-fold. With the more active PTPase from *Yersinia*, the relative enzymatic versus uncatalyzed rates are 1.4×10^{11} for the native enzyme and 5.9 × 10^7 for the mutant D356N (Zhang et al., 1994).

Role of Desolvation. The hydrolysis of dianions is accelerated by several hundredfold by the addition of dipolar aprotic solvents to the aqueous medium (Kirby & Varvoglis, 1967). In anhydrous *tert*-butanol at 30 °C, the reaction of pNPP dianion is 4 orders of magnitude faster than the aqueous hydrolysis rate. By comparison, the monoanion of pNPP undergoes aqueous hydrolysis 2 orders of magnitude faster than the dianion (Kirby & Varvoglis, 1967). Thus, desolvation can provide greater rate acceleration than proton transfer to the leaving group. In the enzyme−substate complex (Figure 3), the phosphoryl group has been stripped of its solvation shell and has interactions with the guanidinium group of Arg-130 and with backbone amide NH groups. Although it is difficult to quantify where this environment will lie in the continuum between those of water and *tert*-butanol, desolvation could provide a significant portion of the driving force for catalysis in this family of enzymes.

Role of the Cys Anion. In solvent mixtures, both phosphate monoester monoanions and dianions act as very nonselective phosphorylating agents (Kirby & Varvoglis, 1967; Chanley & Feageson, 1963). In aqueous solution, the hydrolysis rate of the dianion of pNPP is constant from pH 9 to pH 13, indicating that phosphoryl transfer is not enhanced by the presence of the more powerful nucleophile hydroxide versus water as the phosphoryl acceptor (Kirby & Jencks, 1965). Both of these behaviors are expected from the dissociative nature of the mechanism, and also from the fact that approach of a negatively charged nucleophile should be disfavored by charge repulsion.[2] This explains why hydroxide ion attacks the dianion of pNPP at C-1 of the aromatic ring of pNPP to give nucleophilic aromatic substitution instead of phosphoryl transfer to hydroxide (Kirby & Jencks, 1965).

These characteristics are reversed in the reactions of VHR and of other PTPases which require that the Cys nucleophile be deprotonated to form the thiolate anion for activity. Replacement of Cys by Ser results in inactivation. Based upon what is known of phosphoryl transfer from phosphate monoesters in solution, it is noteworthy that an enzymatic reaction should show dependence on an anionic nucleophile. The unimportance of nucleophilic pK_a in phosphoryl transfers from monoesters in solution results from the very small degree of bond formation in the transition state, and the fact that the principal driving force arises from electron donation from the nonbridge oxygen atoms (Kirby & Jencks, 1965). If the enzyme were accelerating the reaction by using the anionic nucleophile to enhance nucleophilic participation over what occurs in solution, a more associative transition-state structure would result. However, the isotope effect data show the transition state remains dissociative with no more nucleophilic participation than in the uncatalyzed reaction.

An advantage of an anionic nucleophile in the enzymatic reaction is that it obviates the need for subsequent (or concomitant) proton removal in order to form a stable product. In solution, phosphoryl transfer to a neutral alcohol requires that the nucleophile shed its proton in order to form a stable product. In solution, this can happen rapidly via a hydrogen bonding network with the solvent, while in the protein phosphatase enzymes this would be hindered by the position of the Cys nucleophile at the bottom of the active site cavity and the absence of a basic residue in the vicinity.

Another possible catalytic role for the Cys anion is to provide additional driving force for leaving group expulsion via charge repulsion. In the Michaelis complex (Figure 3), the substrate is positioned such that the thiolate and the leaving group are both apical, at opposite sides of a trigonal bipyramidal arrangement (Jia et al., 1995; Yuvaniyama et al., 1996). Positioning of the phosphoryl group is achieved by interactions with conserved arginine and amide N−H groups. In this position, interactions between the thiolate and the nonbridge oxygens are minimized while those with the phosphorus atom are maximized. The major binding interactions with the polypeptides which are the natural substrates of these enzymes occur in a shallow groove at the surface of the enzyme, and provide the binding energy needed to insert the phosphorylated residue into the active site pocket. Electron donation (or charge repulsion) from the nonbridge oxygen atoms of the dianion provides the main driving force for the uncatalyzed reactions of monoesters. In the Michaelis complex for the enzymatic reaction, the substrate is brought into proximity with the thiolate anion such that it could effectively serve as a third anionic nonbridge atom assisting in leaving group expulsion. The increase in nonbridge P−O bond order revealed by the $^{18}(V/K)_{\text{nonbridge}}$ isotope effects in mutants lacking the general acid demonstrates that the negative charge of the thiolate anion is in sufficient proximity to the substrate that its negative charge is felt by the phosphoryl group. This effect has also been observed in the protein-tyrosine phosphatases YOP and PTP1 (Hengge et al., 1995) and may prove to be a common feature of this family of enzymes.

Conclusions. The transition-state structure of the dual-specific VHR reaction is similar to those found with the PTPases YOP and PTP1 and to that of phosphoryl transfer in solution. These reactions all proceed through highly dissociative transition states with a large degree of bond cleavage to the leaving group, a metaphosphate-like structure of the transferring phosphoryl group, and very little bond formation to the nucleophile. In mutants lacking general acid assistance to the leaving group, the reactions are also characterized by nearly complete bond cleavage to the leaving group; however, the transition-state bond order between phosphorus and the nonbridge oxygen atoms is measurably less than in the solution reaction of the dianion. The most likely cause is charge repulsion between the transferring anionic phosphoryl group and the thiolate and leaving group anions, resulting in greater dispersal of negative charge into the nonbridge oxygen atoms. This effect is greater when the pK_a of the nucleophile is increased. The alternative explanation that the decreased nonbridge P−O bond order results from greater nucleophilic participation in a less dissociative transition state cannot be ruled out by the data. Electrostatic interaction of the active site thiolate with the anionic substrate may be an important important driving force for the enzymatic reaction, together with desolvation of the substrate and general acid catalysis.

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[2] While the electrostatic repulsion between anionic nucleophiles and phosphorylated pyridine monoanions is only moderate (Herschlag & Jencks, 1989b), that between anionic nucleophiles and phosphate dianions should be considerably larger. As a result, while hydroxide reacts with phosphorylated pyridine monoanions by nucleophilic attack at phosphorus, hydroxide reaction with pNPP dianion results in nucleophilic aromatic substitution instead of phosphoryl transfer (Kirby & Jencks, 1965).

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