Nature of the Transition State of the Protein-Tyrosine Phosphatase-Catalyzed Reaction†

Alvan C. Hengge,*,‡ Gwendolyn A. Sowa,‡ Li Wu,§ and Zhong-Yin Zhang*,§

Institute for Enzyme Research, University of Wisconsin, 1710 University Avenue, Madison, Wisconsin 53705, and the Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received August 16, 1995; Revised Manuscript Received September 20, 1995⊗

ABSTRACT: The dephosphorylation of *p*-nitrophenyl phosphate by *Yersinia* protein-tyrosine phosphatase (PTPase) and by the rat PTP1 has been examined by measurement of heavy-atom isotope effects at the nonbridge oxygen atoms [$^{18}(V/K)_{nonbridge}$], at the bridging oxygen atom [$^{18}(V/K)_{bridge}$], and the nitrogen atom in the leaving group $^{15}(V/K)$. The effects were measured using an isotope ratio mass spectrometer by the competitive method and thus are effects on *V/K*. The results for the *Yersinia* PTPase and rat PTP1, respectively, are 1.0142 ± 0.0004 and 1.0152 ± 0.0006 for $^{18}(V/K)_{bridge}$; 0.9981 ± 0.0015 and 0.9998 ± 0.0013 for $^{18}(V/K)_{nonbridge}$; and 1.0001 ± 0.0002 and 0.9999 ± 0.0003 for $^{15}(V/K)$. The magnitudes of the isotope effects are similar to the intrinsic values measured in solution, indicating that the chemical step is rate-limiting for *V/K*. The transition state for phosphorylation of the enzyme is dissociative in character, as is the case in solution. Binding of the substrate is rapid and reversible, as is the binding-induced conformational change which brings the catalytic general acid into the active site. Cleavage of the P−O bond and proton transfer from the general acid Asp to the leaving group are both far advanced in the transition state, and there is no development of negative charge on the departing leaving group. Experiments with several general acid mutants give values for $^{18}(V/K)_{bridge}$ of around 1.0280, $^{15}(V/K)$ of about 1.002, and $^{18}(V/K)_{nonbridge}$ effects of from 1.0007 to 1.0022. These data indicate a dissociative transition state with the leaving group departing as the nitrophenolate anion but suggest more nucleophilic participation than in the solution reaction.

There is growing appreciation of the biological importance of protein-tyrosine phosphatases (PTPases)[1] in signal transduction (Fischer et al., 1991; Charbonneau & Tonks, 1992; Walton & Dixon, 1993). Together with the protein tyrosine kinases (PTKs), the PTPases control the level of tyrosine phosphorylation on cellular proteins. PTPases constitute a growing family of enzymes that rival PTKs in terms of structural diversity and complexity. We have used heavy-atom kinetic isotope effects to learn more about the mechanism of catalysis and to study the transition-state structure of the rate-limiting step of two members of this enzyme class, the *Yersinia* PTPase and the rat PTP1, with the substrate *p*-nitrophenyl phosphate (*p*NPP). The *Yersinia* PTPase is a virulence determinant for the pathogenic bacteria responsible for the bubonic plague (Guan & Dixon, 1990). Rat PTP1 is the structural homologue of the human PTP1B (Guan et al., 1990). Heavy-atom kinetic isotope effects were also determined for the reaction catalyzed by the mutant PTPases in which the general acid Asp residue has been replaced with either Asn or Ala. The data with the mutant enzymes give information about the catalytic role of the general acid as well as revealing the mechanistic and transition state consequences of its removal.

All members of the PTPase family, from bacteria to mammals, share significant sequence homology within a 250 residue span of the catalytic domain (Zhang et al., 1994a). The hallmark of the PTPase family is the active site sequence (H/V)**C**(X)$_5$**R**(S/T) in the catalytic domain called the PTPase signature motif (Zhang et al., 1994b). The structural features of the PTPase signature motif, which forms the active site phosphate-binding loop, are conserved from bacteria to mammals (Stuckey et al., 1994; Barford et al., 1994). An invariant Arg residue in the signature motif plays an important role in substrate binding and transition state stabilization (Zhang et al., 1994b). The invariant Cys residue has been shown to be essential for the PTPase activity and the formation of a covalent phosphoenzyme intermediate (Guan & Dixon, 1991; Wo et al., 1992; Cho et al., 1992).

An invariant Asp residue (Asp356 in the *Yersinia* PTPase) has been suggested to function as a general acid in the catalytic mechanism of the PTPases by a combination of site-directed mutagenesis and pH-rate profile analysis and is believed to protonate the leaving group (Zhang et al., 1994a). In the *Yersinia* PTPase, Asp356 is found on a flexible loop which undergoes a major conformational change upon binding of tungstate (Stuckey et al., 1994). This loop movement brings Asp356 within 3.5 Å of an oxygen atom of the tungstate bound to the active site which is structurally homologous to the scissile oxygen of a phosphotyrosine substrate. Similarly, binding of either phosphotyrosine or phosphotyrosine-containing peptide to the Cys215 to Ser mutant mammalian PTPase, PTP1B, causes a conformational

† This work was supported by grants from the NIH to A.C.H. (GM 47297) and to Z.-Y.Z. (DRTC 5P60 DK20541-17). G.A.S. was supported by NIH Grant GM 18938 to W. W. Cleland.

* Authors to whom correspondence should be addressed.

‡ University of Wisconsin.

§ Albert Einstein College of Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] Abbreviations: PTPase, protein-tyrosine phosphatase; *p*NPP, *p*-nitrophenyl phosphate. The notation used to indicate isotope effects is that of Northrop (1977) in which a leading superscript is used, i.e., ^{15}k and ^{18}k refer to an ^{15}N isotope effect (k_{14}/k_{15}) and ^{18}O isotope effect (k_{16}/k_{18}), respectively; similarly, $^{18}(V/K)$ represents the ^{18}O isotope effect on *V/K*, or $(V_{16}/K_{16})/(V_{18}/K_{18})$.

0006-2960/95/0434-13982$09.00/0

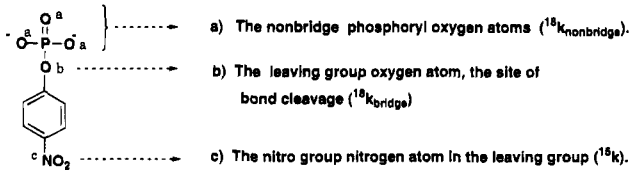


FIGURE 1: *p*NPP substrate showing the positions of isotope effect measurement.

change of an equivalent surface loop that brings the corresponding Asp181 into the catalytic site (Jia et al., 1995).

There is much evidence to indicate that in solution phosphate monoesters hydrolyze via a dissociative mechanism (Butcher & Westheimer, 1955; Bunton et al., 1967; Kirby & Varvoglis, 1967; Gorenstein et al., 1977) involving an unsymmetrically "exploded" metaphosphate-like transition state where bond formation to the incoming nucleophile is minimal and bond breaking between phosphorus and the leaving group is substantial (Bourne & Williams, 1984; Skoog & Jencks, 1984; Herschlag & Jencks, 1989). Secondary ^{18}O kinetic isotope effects in the nonbridging positions of the phosphate group (Weiss et al., 1986; Cleland, 1990) and primary ^{18}O kinetic isotope effects in the leaving group oxygen (Gorenstein et al., 1977; Hengge et al., 1994) are also consistent with a dissociative transition state for nonenzymatic hydrolysis of phosphate monoesters. It has been suggested that an enzyme-catalyzed phosphoryl transfer reaction may prefer an associative mechanism since an enzyme could stabilize the increased negative charge in the transition state (Knowles, 1980; Hasset et al., 1982).

Transition-state structures can be probed in detail using kinetic isotope effects. In phosphoryl transfer reactions the primary ^{18}O isotope effect in the bridging oxygen atom, $^{18}k_{bridge}$, gives an indication of the extent of bond cleavage to the leaving group. The secondary isotope effect in the nonbridge oxygen atoms, $^{18}k_{nonbridge}$, reveals whether the transition state is dissociative (metaphosphate-like), or associative, resembling a pentavalent phosphorane. When the leaving group is *p*-nitrophenol, the ^{15}N isotope effect 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. The sites in the *p*NPP substrate where isotope effects were measured are shown graphically in Figure 1. The isotope effects have previously been measured for the well-characterized solution reactions of both the monoanion and the dianion of *p*NPP (Hengge et al., 1994). Comparison of the isotope effects on the PTPase-catalyzed dephosphorylation of *p*NPP with the solution data reveals a detailed picture of the transition state structure of the enzymatic reaction.

MATERIALS AND METHODS

Materials. Alkaline phosphatase from *Escherichia coli*, type III, and natural abundance *p*NPP were purchased from Sigma. Succinic acid and 3,3-dimethyl glutaric acid were purchased from Aldrich.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using the Muta-Gene *in vitro* Mutagenesis kit from Bio-Rad. The Asp356 to Asn (D356N) mutation was made in an early study (Zhang et al., 1994a). The oligonucleotide primer used for D356A was ATTGGCCCGCTCAGACCGC. The underlined base indicate the change from the naturally occurring nucleotides. The plasmid pUC118-PTP1U323 encoding the first 322 amino acid residues of the rat PTP1 that contains the entire PTPase domain (Guan & Dixon, 1991b) was used to make single-stranded DNA for site-directed mutagenesis. The plasmid pUC118-PTP1U323 was created by ligation of a *Xba*I/*Bam*HI fragment containing the first 322 amino acids of PTP1 from pET3d-PTP1U323 (a generous gift from Nick Santoro) into pUC118. The catalytic domain of PTP1 resides between residues 1 and 322 (Guan & Dixon, 1991a) and is 97% identical to the corresponding 322 residues of the human PTP1B. The oligonucleotide primer used for the D181N substitution was ACCTGGCCTAACTTTGGAG. All of the mutations were verified by DNA sequencing.

Expression and Purification of the Recombinant Enzymes. The wild-type and the mutant *Yersinia* PTPases were expressed in *E. coli* and purified to homogeneity as described previously (Zhang et al., 1992). The expression vector pET3d-PTP1U323 was created as follows: an *Eco*RI/*sac*I PTP1 fragment taken from pGEX-KG (Guan & Dixon, 1991b) was ligated into pUC18. This PTP1 fragment was then removed from the resulting pUC18 construct as a *Xho*I/*Bam*HI fragment and was ligated in a triple ligation to an *Nco*I/*Xho*I fragment obtained from pPTP1 [cDNA vector (Guan et al., 1990)] and a pET3d vector previously digested with *Nco*I/*Bam*H1. To purify a large quantity of rat PTP1U323, an overnight culture (10 mL) was grown from a single colony of pET3d-PTP1U323 and was used to inoculate 1 L of 2×YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing 100 μg/mL ampicillin. The culture was grown at 37 °C to an optical density of 0.6−0.9 at 600 nm, induced with 0.4 mM isopropyl β-D-thiogalactoside, and grown for an additional 4−5 h at 37 °C. The cells were harvested by centrifugation at 5000 rpm (Sorvall GSA rotor) for 10 min. The resulting pellet was then resuspended in 40 mL of 25 mM Tris, 25 mM NaCl, 1 mM DTT, and 2 mM EDTA, pH 7.4 (buffer A). Cells were lysed by two passages through a French press, maintaining pressures above 1200 psi. The soluble fraction was isolated by centrifugation of the lysates at 15 000 rpm (Sorvall SS-34 rotor) for 20 min at 4 °C. Solid ammonium sulfate was added slowly to the resultant clear supernatant to 1.7 M. The lysate was centrifuged again as above, and the large protein precipitate was discarded. The clear supernatant was then applied to a phenyl Sepharose column (40 mL bed volume, Sigma) which had been equilibrated in 25 mM Tris, pH 7.4, 1 mM DTT, and 2 mM EDTA (buffer B) plus 1.7 M ammonium sulfate at room temperature. The column was extensively washed (400 mL) with the same buffer. The enzyme was eluted with a 200 mL linear gradient consisting of buffer B + 1.7 M ammonium sulfate in one chamber and buffer B in the other chamber. The fractions with PTPase activity were pooled and dialyzed overnight against buffer B at 4 °C. The dialyzed sample was loaded onto a CM Sephadex column (50 mL bed volume), which was pre-equilibrated with buffer B at 4 °C. The column was again washed with buffer B until the eluent had a zero optical density at 280 nm. PTP1U323 was eluted from this column with a 200 mL salt gradient consisting of buffer B in one chamber and buffer B plus 250 mM NaCl in the other. The D181N mutant PTP1 was obtained by the same procedure described above.

Synthesis of Compounds. [^{14}N]-*p*-Nitrophenyl phosphate, [^{15}N, nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate, [^{14}N]-*p*-nitrophenol, and [^{15}N,^{18}O]-*p*-nitrophenol were synthesized as previously described (Hengge et al., 1994). The two isotopic

isomers of *p*-nitrophenol were mixed in proportions to reconstitute the natural abundance of ^{15}N and then phosphorylated to produce *p*-nitrophenyl phosphate by the same method as for the other isotopic forms of *p*-nitrophenyl phosphate.

Isotope Effect Determinations. Isotope effect experiments run at pH 5.0, 6.0, and 7.0 were performed respectively in 100 mM acetate, succinate, and 3,3-dimethyl glutarate buffers, with 1 mM EDTA. The reactions run with the wild-type enzymes were performed at 25 °C, while those with the mutant enzymes were run at 35 °C in order to shorten reaction times and conserve enzyme. In each experiment approximately 100 μmol of *p*-nitrophenyl phosphate was dissolved in either 25 mL (with reactions with wild-type enzymes) or 10 mL (with mutant enzymes) of buffer, and sufficient enzyme was added to cause reaction half-life of about 5−6 h. Reactions were run in triplicate and were stopped at different fractions of reaction, which typically ranged from 20 to 70%. Reaction progress was followed by assaying for free *p*-nitrophenol by adding an aliquot of the reaction mixture to 0.4 N sodium hydroxide and reading the absorbance at 400 nm. Reactions were stopped by cooling the reaction flask in ice and extracting the *p*-nitrophenol with diethyl ether (3 × 25 mL). After the extracts were dried over magnesium sulfate, the ether was removed by rotary evaporation.

For determination of the fraction of reaction, before extraction with ether an aliquot of the chilled reaction mixture was diluted into Tris buffer at pH 9 and assayed for *p*-nitrophenol, treated with alkaline phosphatase overnight, and then assayed again. Control experiments showed that the fraction of reaction determined by this method agreed with that determined by separately assaying *p*-nitrophenol in the isolated product and in the residual substrate after complete hydrolysis.

The aqueous layer which contained the unreacted *p*-nitrophenyl phosphate was briefly subjected to rotary evaporation to removed dissolved ether and completely hydrolyzed by treatment with approximately 3 units of alkaline phosphatase at pH 9 for 12 h. The *p*-nitrophenol thus produced from the remaining substrate was isolated by acidification to pH 6 and ether extraction. The *p*-nitrophenol samples were further purified by sublimation and combusted to produce nitrogen gas which was analyzed by isotope ratio mass spectrometry, by methods that have been described (Hengge et al., 1994).

Data Analysis. 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*. Equation 2 was used to calculate the observed isotope effect from R_s and R_o.

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

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

R_o, the nitrogen isotopic ratio in the starting material, was determined from nitrogen obtained from combustion of samples of *p*-nitrophenyl phosphate substrate and, as a control, by completely hydrolyzing samples of the substrate and combustion of the nitrophenol produced and isolated by the same method used in the isotope effect experiments. The isotopic ratios obtained from both methods were the same within experimental error, showing that no isotopic fractionation occurs during the procedures used to recover *p*-nitrophenol.

The ^{18}O isotope effects were measured by the remote-label method (O'Leary & Marlier, 1979), as described for the solution reactions of pNPP (Hengge et al., 1994). These experiments yield an observed isotope effect which is the product of the effect due to ^{15}N substitution and that due to the ^{18}O. 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 (Caldwell et al., 1991).

Table 1: Kinetic Isotope Effects with *p*NPP

pH	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
	PTPase Reactions		
	PTP1		
5.5 (optimum)	1.0001 ± 0.0002	1.0142 ± 0.0004	0.9981 ± 0.0015
6.0	1.0002 ± 0.0004	1.0170 ± 0.0020	
7.0	1.0006 ± 0.0006	1.0181 ± 0.0006	1.0002 ± 0.0013
D181N (pH 6)	1.0019 ± 0.0002	1.0278 ± 0.0017	1.0018 ± 0.0003
	Yersinia PTPase		
5.0 (optimum)	0.9999 ± 0.0003	1.0152 ± 0.0006	0.9998 ± 0.0013
6.0	0.9999 ± 0.0003	1.0160 ± 0.0015	1.0001 ± 0.0013
D356N (pH 6)	1.0024 ± 0.0005	1.0275 ± 0.0016	1.0022 ± 0.0005
D356A (pH 6)	1.0022 ± 0.0003	1.0274 ± 0.0008	1.0007 ± 0.0005
	Solution Reactions[a]		
pH	^{15}k	$^{18}k_{bridge}$	$^{18}k_{nonbridge}$
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] From Hengge et al. (1994).

RESULTS

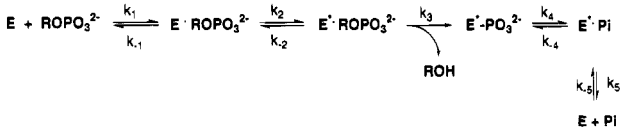
The isotope effects are listed in Table 1 with their standard errors. The ^{18}O isotope effects have been corrected for the ^{15}N effect and for levels of isotopic incorporation. At least six determinations were made of each isotope effect. The effects calculated from R_p and R_o, and from R_s and R_o, gave excellent agreement in all cases, as did those measured at differing fractions of reaction, and were averaged together to give the values reported in the table. Experiments were performed with the wild-type enzymes at their optimal pH (5.5 for PTP1 and 5.0 for the *Yersinia* enzyme) and at higher pH (6.0 with both enzymes and also at pH 7.0 with PTP1) where chemistry is slower (Zhang et al., 1994c; Zhang, 1995). Experiments with the general acid mutants were performed at pH 6.0.

Because the substrate for the enzymes is the dianion of pNPP, the observed values for $^{18}(V/K)_{nonbridge}$ were corrected for the isotopic fractionation which affects the proportion of the isotopic isomers present as the dianion at a given pH (Hengge et al., 1994).

All isotope effects were measured by the competitive method using an isotope ratio mass spectrometer, which is by far the most accurate method for the measurement of the comparatively small magnitudes of heavy-atom isotope effects. Since the competitive method gives effects on *V/K*, the isotope effects reported here are those on the first part of the mechanism through the first irreversible step, shown as k_3 in Scheme 1.

The isotope effects for the uncatalyzed solution hydrolysis reactions of *p*NPP, determined in a previous study, have been

Scheme 1



included in Table 1 for comparison with the PTPase data. The aqueous hydrolyses were performed at 95 °C, and an approximation of the temperature effect on the isotope effects was made using the equation ln(IE at 30 °C) = (368 K/303 K) ln(IE at 95 °C). The effects for the reactions in solution listed in Table 1 are the values corrected to 30 °C. In the present study, the experiments with wild-type enzymes were performed at 25 °C, and those with the mutants at 35 °C. The magnitudes of the corrections for this 10 °C difference are smaller than the experimental errors and are not listed in the table.

DISCUSSION

Factors Influencing the Isotope Effects. The PTPase mechanism is shown in Scheme 1. The mechanism shown includes the conformational change which occurs upon binding of substrate. Hydrolysis of the phosphoenzyme intermediate is the rate-limiting step for V_{max}. The isotope effects in this study were measured using the competitive method which yields isotope effects on *V/K* (Northrop, 1982). Thus the isotope effects reported here are sensitive only to steps in the mechanism up to and including the first irreversible step which is release of the *p*-nitrophenol product, shown as k_3 in Scheme 1, and are insensitive to all subsequent steps.

The interpretation of kinetic isotope effects in an enzymatic reaction relies on knowledge of the degree to which a chemical step is rate-limiting. If a nonchemical step such as binding of substrate or the enzymatic conformational change described earlier, which brings the catalytic general acid Asp near the substrate, is partially or fully rate-limiting, then isotope effects on the chemical step will be suppressed and their magnitudes will not reflect the true structure of the transition state.

If only the chemical step (k_3) in *V/K* is isotope sensitive, the expression for the observed ^{18}O isotope effect from the mechanism shown is given by eq 3. The possibility of an isotope-sensitive binding step for the nonbridge oxygen atoms arising from their proximity to an arginine in the active site is discussed later. The conformational change will not be isotope sensitive since no motions of the substrate are involved.

$$\text{observed } {}^{18}(V/K) = ({}^{18}k_3 + c_f)/(c_f + 1) \qquad (3)$$

In this expression the commitment factor c_f is equal to $(k_3/k_{-2})(1 + k_2/k_{-1})$. The ratio k_3/k_{-2} reflects the fate of the enzyme−substrate complex after the conformational change. If the conformational change is not freely reversible, the substrate will be committed to undergo the chemical step and the resulting large value of the ratio k_3/k_{-2} will diminish the observed isotope effects. Similarly the ratio k_2/k_{-1} reflects the partitioning of the initial enzyme−substrate complex. If the substrate is "sticky", this ratio will be large and the isotope effects will be suppressed. The phosphoryl transfer step k_3 should be irreversible, both because *p*-nitrophenol is a very poor inhibitor of PTPases (Zhang et al., 1994c), indicating that its dissociation from the active site is fast, and also because of *p*-nitrophenol's poor nucleophilicity. Since *p*NPP is a slow substrate for these enzymes, with K_m values much higher than for more natural polypeptide substrates (Zhang et al., 1994d), the ratio k_2/k_{-1} should be small.

There are two ways one can ascertain whether or not the chemical step is rate-limiting and the isotope effects observed are the intrinsic ones for the chemical step. The best is to compare the enzymatic isotope effects with those for the same reaction of the identical substrate in solution, where the commitments which often occur in enzymatic reactions are not present. Another is to measure the isotope effects at the pH optimum and at a point well off the optimum, where the rate of the chemical step is slower and the commitment factor may therefore decrease, causing an increase in the observed isotope effect. A large commitment factor can completely abolish isotope effects (that is, the observed isotope effects are unity) even when reaction occurs at a pH value well off the optimum, as occurs in the alkaline phosphatase reaction with the substrate pNPP (Hengge et al., 1994). In cases where commitment factors are smaller but still significant, intrinsic isotope effects may be partially suppressed but measurable at the pH optimum. Changes in pH may then serve to reduce the commitment factor and increase the observed isotope effects. In such cases, the limiting values of the isotope effects at the pH extremes need not (and usually do not) reflect the intrinsic isotope effects on the bond-breaking step (Cook, 1991). Only if significant commitment factors are absent will the full intrinsic isotope effects be expressed at the pH optimum, and the magnitudes of the isotope effects in this case will not increase upon changes in pH.

In the present study, the solution results for the hydrolysis of *p*NPP in water and in *tert*-butanol give us a benchmark for evaluating the magnitudes of the enzymatic effects. In addition, the isotope effects for the wild-type enzymes were measured at the pH optimum and also at higher pH where the chemical step is slower and the commitment, if significant, should be smaller. The isotope effect with the largest magnitude and therefore the most sensitive to the presence of a commitment is $^{18}(V/K)_{bridge}$. The values from experiments with the wild type enzymes for $^{18}(V/K)_{bridge}$ should be compared with the value of $^{18}k_{bridge}$ for the monoanion reaction in solution, since in each of these reactions the leaving group is protonated and is expelled as the neutral species, in contrast to the dianion reaction where it leaves as the *p*-nitrophenolate anion. The magnitude of this isotope effect will be large for the chemical step of bond cleavage but will be negligible for the binding step (which involves the nonbridge oxygens) or for an enzymic conformational change. To the extent that either of the latter two steps are partially rate-limiting, the observed magnitude of $^{18}(V/K)_{bridge}$ will be diminished relative to the value in solution.

Conclusions from the Isotope Effects. The value of $^{18}(V/K)_{bridge}$ for both enzymes is about 1.5%, compared to that of the monoanion reaction in water which is calculated to be about 1.1% at 30 °C. The PTPase values do not change significantly with pH (the slight increase with pH that occurs with PTP1 is caused by other effects and is discussed later). It is therefore likely that the chemical step of cleavage of the P−O bond is rate-limiting for *V/K* even at the pH optimum, and that the isotope effects expressed are the

intrinsic ones for this chemical step.[2] This also indicates that the conformational change which brings the loop with the general acid Asp into proximity with the substrate must be freely reversible in solution, at least with the substrate *p*NPP.

The isotope effect data for the solution reaction of the monoanion indicate a transition state where the proton transfer from the nonbridge oxygen to the leaving group oxygen is extensive but has not quite kept up with bond cleavage. This is indicated by the presence of the small ^{15}k isotope effect which indicates that a small amount of negative charge is delocalized in the aromatic ring of the leaving group (Hengge et al., 1994). In the enzymatic reactions the $^{15}(V/K)$ effect is completely abolished, indicating complete charge neutralization of the leaving group by the general acid in the transition state. Thus proton transfer to the departing leaving group at the transition state has fully compensated for P−O bond cleavage and thus is sufficient to prevent buildup of negative charge. The $^{18}k_{\text{nonbridge}}$ isotope effect in the solution monoanion reaction reflects the deprotonation of a nonbridge oxygen atom. The nonbridge oxygen atoms are not involved in proton transfers in the enzymatic reaction, where the substrate is the dianion and the proton transferred to the leaving group comes from the general acid Asp.

The large magnitudes of $^{18}(V/K)_{\text{bridge}}$ in the *Yersinia* PTPase and PTP1 reactions indicate a dissociative transition state with a large degree of bond cleavage in the transition state, similar to the solution reaction. The value of $^{18}(V/K)_{\text{bridge}}$ is affected both by bond cleavage, which produces a normal isotope effect, and by protonation, which produces an inverse effect. In an associative transition state bond cleavage to the leaving group is less advanced, and its contribution to the magnitude of the isotope effect therefore less. Phosphotriesters exhibit associative transition states, and in the alkaline hydrolysis of the phosphotriester diethyl *p*-nitrophenyl phosphate, $^{18}k_{\text{bridge}}$ has a value of 1.0060 (Caldwell et al., 1991). Protonation of the leaving group in an associative transition state would reduce the overall $^{18}(V/K)_{\text{bridge}}$ effect even below this value. Thus the magnitudes of the PTPase effects are most consistent with a dissociative transition state. Because these isotope effects have contributions from protonation as well as from bond cleavage, it would be unwise to use the $^{18}(V/K)_{\text{bridge}}$ effects to calculate a specific percentage of bond cleavage in the transition states of the enzymatic reaction compared to the uncatalyzed solution reaction.

The small, inverse values for $^{18}(V/K)_{\text{nonbridge}}$ in the *Yersinia* PTPase and PTP1 reactions are similar to those for $^{18}k_{\text{nonbridge}}$ in the solution reactions of the dianion, indicating that the central phosphoryl group is similarly metaphosphate-like in structure in the transition state. The small magnitudes of the isotope effects in the nonbridge oxygen atoms also lend support to computational results which suggest that metaphosphate is more accurately portrayed as a resonance hybrid

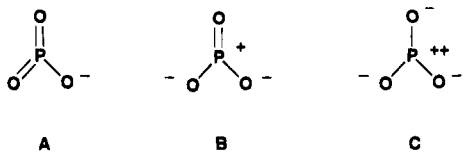


FIGURE 2: Resonance structures for metaphosphate ion.

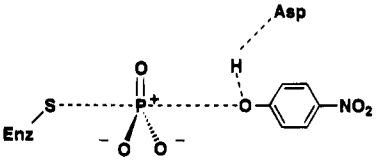


FIGURE 3: Transition-state structure for PTPase-catalyzed dephosphorylation of *p*NPP inferred from the isotope effect data.

of structures B and C in Figure 2, as opposed to the classical representation A (Rajca et al., 1987; Horn & Ahlrichs, 1990). With this in mind, the transition state for the PTPase reactions can be said to resemble the structure in Figure 3. The P−O bond to the leaving group is largely broken, proton transfer to the leaving group oxygen is correspondingly advanced such that the departing phenol has no charge, and the central phosphoryl group resembles metaphosphate in structure. These experiments give no direct measure of the degree of bond formation to the nucleophile in the transition state. However, the close agreement of the isotope effects to those of the solution reactions argue that the degree of bond formation is very small, as it is in solution.

The similarity of the enzymatic values for $^{18}(V/K)_{\text{nonbridge}}$ to the solution $^{18}k_{\text{nonbridge}}$ also indicates that no isotope effect arises from binding of pNPP to the enzyme active site. Since both isotopic isomers of the substrate are present in these competitive experiments, an isotope effect on binding will result in isotopic discrimination in the bound versus unbound substrate. The observed isotope effect will be the product of the effect on substrate binding and that on the chemical step. Binding involves positioning of the phosphoryl nonbridge oxygens in close proximity with an Arg residue. Protonation of a nonbridge oxygen atom results in an inverse isotope effect of about 1.6% in phosphate esters (Knight et al., 1986). The lack of any effect indicates that there is no significant sharing of a proton between the nonbridge oxygens of the substrate and the arginine in the phosphate binding pocket and that the interaction is electrostatic in nature. Such interactions of phosphate with metal cations such as magnesium similarly do not result in isotopic fractionation (Jones et al., 1991).

The isotope effect data do not themselves rule out a pre-equilibrium protonation of the leaving group at the bridge oxygen atom followed by rate-limiting loss of the leaving group. However, active site mutants where the nucleophilic Cys is replaced by Ser are inactive (Guan & Dixon, 1990), which argues against a pre-equilibrium transfer. Such a step would create an extremely electrophilic and labile species with which the serine hydroxyl, protonated or not, would be expected to react. An analogous precedent is the aqueous monoanion hydrolysis, where water is the nucleophile which attacks the similar species which is activated by proton transfer to the leaving group (in this case, probably concurrent with bond cleavage). It is thus much more likely that some small degree of nucleophilic participation by the thiolate is necessary to initiate reaction and that proton transfer accompanies cleavage of the P−O bond.

[2] A reviewer raised the question of whether the isotope effect data could be simply be interpreted in terms of a commitment of about 1 with the wild-type enzymes, which is reduced with the slower mutants, thus explaining the larger isotope effects observed for $^{18}(V/K)_{\text{bridge}}$ with the mutants. This possibility is ruled out by the other isotope effects, $^{18}(V/K)_{\text{nonbridge}}$ and $^{15}(V/K)$. The presence of a commitment will diminish all isotope effects proportionally, so if $^{18}(V/K)_{\text{bridge}}$ is reduced by about half in the reaction with wild-type enzyme, then the same fractional reduction is predicted to occur in $^{18}(V/K)_{\text{nonbridge}}$ and $^{15}(V/K)$. As the data in Table 1 show, this is not what is observed.

In the *Yersinia* PTPase mutants D356N and D356A, and in the PTP1 mutant D181N, the general acid has been removed. With this loss of general acid catalysis the leaving group must depart as the *p*-nitrophenolate anion. This is reflected in the appearance of a sizeable $^{15}(V/K)$ effect, indicating delocalization of negative charge into the aromatic ring, and in the increased magnitude of $^{18}(V/K)_{bridge}$. Protonation of *p*-nitrophenol causes an inverse equilibrium isotope effect of 1.53% (Hengge & Hess, 1994), and removal of this inverse contribution to $^{18}(V/K)_{bridge}$ results in larger normal values compared to those observed in the wild-type enzymatic reactions. In the D356N and D181N mutants one may expect the possibility of hydrogen bonding between the departing phenolate and the asparagine, but the isotope effect data give no indication that such an interaction occurs in the transition state.

Surprisingly, the values for $^{18}(V/K)_{nonbridge}$ become slightly normal in reactions with the mutants. While the values are well below those seen in reactions with associative transition states where values of from 1.0056 to 1.0200 have been seen (Caldwell et al., 1991), the central phosphoryl group is measurably less metaphosphate-like in these reactions than in those with the wild-type enzyme or those in solution. Leaving group departure is still far advanced, and presumably the difference in $^{18}(V/K)_{nonbridge}$ is caused by an increase in nucleophilic interaction between sulfur and phosphorus, which can be rationalized by the need for a greater "push" to expel the leaving group in the absence of general acid assistance.

This result also explains the slight rise with increasing pH of the leaving group isotope effects $^{18}(V/K)_{bridge}$ and $^{15}(V/K)$ with the wild-type PTP1. The rate of the D181N mutant reaction is only about two orders of magnitude slower relative to the maximum of the wild-type. Thus as the reaction is monitored at higher pH values where the wild-type rate is slowed, some enzymatic reaction occurs from the incorrectly protonated form of the enzyme, without general acid catalysis. In these cases the observed effects are products of those for the two processes, and so are increased in magnitude in proportion to the contribution of reaction which occurs when Asp181 is deprotonated and the leaving group departs as the anion.

In summary, our results demonstrate that (a) P−O bond cleavage is rate-limiting for the *V/K* portion of the mechanisms of both enzymes, and that substrate binding and the conformational change induced by binding are rapid and reversible, (b) the transition state is similar for both enzymes and is highly dissociative, similar to that in solution, and (c) the proton from the general acid is largely transferred to the bridge oxygen in the transition state.

ACKNOWLEDGMENT

We thank W. W. Cleland for useful comments on this work.

REFERENCES

Barford, D., Flint, A. J., & Tonks, N. K. (1994) *Science 263*, 1397−1404.
Bourne, N., & Williams, A. (1984) *J. Am. Chem. Soc. 106*, 7591−7596.
Bunton, C. A., Fendler, E. L., Humeres, E., & Yang, K.-U. (1967) *J. Org. Chem. 32*, 2806−2811.
Butcher, W. W., & Westheimer, F. H. (1955) *J. Am. Chem. Soc. 77*, 2420−2424.
Caldwell, S. R., Raushel, F. M., Weiss, P. M., & Cleland, W. W. (1991) *Biochemistry 30*, 7444−7450.
Charbonneau, H., & Tonks, N. K. (1992) *Annu. Rev. Cell. Biol. 8*, 463−493.
Cho, H., Krishnaraj, R., Kitas, E., Bannwarth, W., Walsh, C. T., & Anderson, K. S. (1992) *J. Am. Chem. Soc. 114*, 7296−7298.
Cleland, W. W. (1990) *FASEB J. 4*, 2899−2905.
Cook, P. F. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) p 243, CRC Press, Cleveland, OH.
Fischer, E. H., Charbonneau, H., & Tonks, N. K. (1991) *Science 253*, 401−406.
Gorenstein, D. G., Lee, Y.-G., & Kar, D. (1977) *J. Am. Chem. Soc. 99*, 2264−2267.
Guan, K. L., & Dixon, J. E. (1990) *Science 249*, 553−556.
Guan, K. L., & Dixon, J. E. (1991a) *J. Biol. Chem. 266*, 17026−17030.
Guan, K. L., & Dixon, J. E. (1991b) *Anal. Biochem. 192*, 262−267.
Guan, K. L., Haun, R. S., Watson, S. J., Geahlen, R. L., & Dixon, J. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1501−1505.
Hasset, A., Blättler, W., & Knowles, J. R. (1982) *Biochemistry 21*, 6335−6340.
Hengge, A. C., & Hess, R. A. (1994) *J. Am. Chem. Soc. 116*, 11256−11263.
Hengge, A. C., Edens, W. A., & Elsing, H. (1994) *J. Am. Chem. Soc. 116*, 5045−5049.
Horn, H., & Alhrichs, R. (1990) *J. Am. Chem. Soc. 112*, 2121−2124.
Herschlag, D., & Jencks, W. P. (1989) *J. Am. Chem. Soc. 111*, 7579−7586.
Jia, Z., Barford, D., Flint, A. J., & Tonks, N. K. (1995) *Science 268*, 1754−1758.
Jones, J. P., Weiss, P. W., & Cleland, W. W. (1991) *Biochemistry 30*, 3534−3639.
Knight, W. B., Weiss, P. M., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 2759−2761.
Kirby, A. J., & Varvoglis, A. G. (1967) *J. Am. Chem. Soc. 89*, 415−423.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877−919.
Northrop, D. B. (1971) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
Northrop, D. B. (1982) *Methods Enzymol. 87*, 616.
O'Leary, M. H., & Marlier, J. F. (1979) *J. Am. Chem. Soc. 101*, 3300−3306.
Rajca, A., Rice, J., Streitweiser, A., & Schaefer, H. (1987) *J. Am. Chem. Soc. 109*, 4189−4192.
Skoog, M. T., & Jencks, W. P. (1984) *J. Am. Chem. Soc. 106*, 7597−7606.
Stuckey, J. A., Fauman, E. B., Schubert, H. L., Zhang, Z.-Y., Dixon, J. E., & Saper, M. A. (1994) *Nature 370*, 571−575.
Walton, K., & Dixon, J. E. (1993) *Annu. Rev. Biochem. 62*, 101−120.
Weiss, P. M., Knight, W. B., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 2761−2762.
Wo, Y.-Y. P., Zhou, M.-M., Stevis, P., Davis, J. P., Zhang, Z.-Y., & Van Etten, R. L. (1992) *Biochemistry 31*, 1712−1721.
Zhang, Z.-Y. (1995) *J. Biol. Chem. 270*, 11199−11204.
Zhang, Z.-Y., Clemens, J. C., Schubert, H. L., Stuckey, J. A., Fischer, M. W. F., Hume, D. M., Saper, M. A., & Dixon, J. E. (1992) *J. Biol. Chem. 267*, 23759−23766.
Zhang, Z.-Y., Wang, Y., & Dixon, J. E. (1994a) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1624−1627.
Zhang, Z.-Y., Wang, Y., Wu, L., Fauman, E., Stuckey, J. A., Schubert, H. L., Saper, M. A., & Dixon, J. E. (1994b) *Biochemistry 33*, 15266−15270.
Zhang, Z.-Y., Malachowski, W. P., Van Etten, R. L., & Dixon, J. E. (1994c) *J. Biol. Chem. 269*, 8140−8145.
Zhang, Z.-Y., Maclean, D., McNamara, D. J., Sawyer, T. K., & Dixon, J. E. (1994d) *Biochemistry 33*, 2285−2290.

BI951943P