Examination of the Transition State of the Low-Molecular Mass Small Tyrosine Phosphatase 1. Comparisons with Other Protein Phosphatases†

Alvan C. Hengge*,‡

Institute for Enzyme Research, University of Wisconsin, 1710 University Avenue, Madison, Wisconsin 53705

Yu Zhao, Li Wu, and Zhong-Yin Zhang*

Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received February 18, 1997; Revised Manuscript Received May 5, 1997⊗

ABSTRACT: The reactions of *p*-nitrophenyl phosphate (*p*NPP) with the low-molecular mass tyrosine phosphatase Stp1 and with the mutants D128N, D128A, D128E, and S18A have been studied by measurement of heavy-atom isotope effects in the substrate. The isotope effects were measured at the nonbridging oxygen atoms [$^{18}(V/K)_{nonbridge}$], at the bridging oxygen atom (the site of bond cleavage) [$^{18}(V/K)_{bridge}$], and at the nitrogen atom in the nitrophenol leaving group [$^{15}(V/K)$]. The results with native Stp1 were 1.0160 ± 0.0005 for $^{18}(V/K)_{bridge}$, 1.0007 ± 0.0001 for $^{15}(V/K)$, and 1.0018 ± 0.0003 for $^{18}(V/K)_{nonbridge}$. The values for $^{18}(V/K)_{nonbridge}$ and $^{15}(V/K)$ differ from those previously measured with other protein-tyrosine phosphatases and from those of the aqueous hydrolysis reaction of *p*NPP. The values indicate that in the transition state of the native Stp1 reaction the leaving group bears a partial negative charge, and there is nucleophilic interaction between the Cys nucleophile, and the phosphoryl group, causing some decrease in the nonbridge P−O bond order. The transition state remains highly dissociative with respect to the degree of bond cleavage to the leaving group. Mutation of the general acid from aspartic acid to glutamic acid slows catalysis but causes no change in the isotope effects and thus does not alter the degree of proton transfer to the leaving group in the transition state. Mutations of this residue to asparagine or alanine give values for $^{18}(V/K)_{bridge}$ of about 1.029, for $^{15}(V/K)$ of about 1.003, and for $^{18}(V/K)_{nonbridge}$ of 1.0010 (D128A) to 1.0024 (D128N). These data indicate a dissociative transition state with the leaving group departing as the nitrophenolate anion and indicate more nucleophilic participation than in the aqueous hydrolysis of the *p*NPP dianion, just as in the native enzyme. The isotope effects with the S18A mutant, in which a hydrogen bonding stabilization of the anionic Cys nucleophile has been removed, were within experimental error of those with the native enzyme, indicating that this alteration has no effect on the transition state for phosphoryl transfer from *p*NPP.

Protein-tyrosine phosphatases (PTPases),[1] working in concert with protein-tyrosine kinases, regulate a variety of cellular functions by controlling the tyrosine phosphorylation state of cellular proteins (Tonks & Neel, 1996). The hallmark of PTPases is the active site sequence (H/V)C-$(X)_5$R(S/T) that forms the phosphate binding loop (Fauman & Saper, 1996). Included in the PTPase superfamily are two additional groups of enzymes, the VH1-like dual-specificity phosphatases and the low-molecular mass (17−18 kDa) tyrosine phosphatases, which share no discernible sequence identity with the PTPases (Zhang, 1997). The only similarities among these three groups of phosphatases are the relative positions of the essential cysteine and arginine residues in the active site sequences. The PTPases hydrolyze only phosphotyrosine residues of polypeptide substrates, while the dual-specificity enzymes process phosphoserine and -threonine residues as well.

Despite the variation in the primary structures and the differences in the active site specificity, all three groups of phosphatases utilize a common mechanism for catalysis (Zhang, 1997), which involves the Cys residue acting as the nucleophile to attack the phosphate ester, forming a thiophosphate intermediate, and the invariant Arg residue playing a role in both substrate recognition and transition state stabilization. The step leading to cysteinyl phosphate formation is facilitated by the protonation of the ester oxygen atom in the leaving group, which is accomplished by the conserved Asp residue acting as a general acid. The Asp residue functions as a general base in the second step by activating a water molecule for the hydrolysis of the phosphoenzyme intermediate, which is further facilitated by the conserved Ser/Thr in the active site sequence. Figure 1 depicts the structural features of the active site of the bovine low-molecular mass tyrosine phosphatase. Indeed, the same spatial arrangement of the Cys, Arg, Ser/Thr, and Asp

†This work was supported by grants from the NIH to A.C.H. (GM 47297) and to Z.-Y.Z. (CA 69202). The instruments used for isotope ratio mass spectrometry were supported by NIH Grant GM 18938 to W. W. Cleland.

* Authors to whom correspondence should be addressed.

‡ Present address: Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0300.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: *p*NPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; Stp1, small tyrosine phosphatase 1; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl) methane. The notation used to express isotope effects is that 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 ^{15}N isotope effect on the rate constant k_3.

S0006-2960(97)00364-4 CCC: $14.00

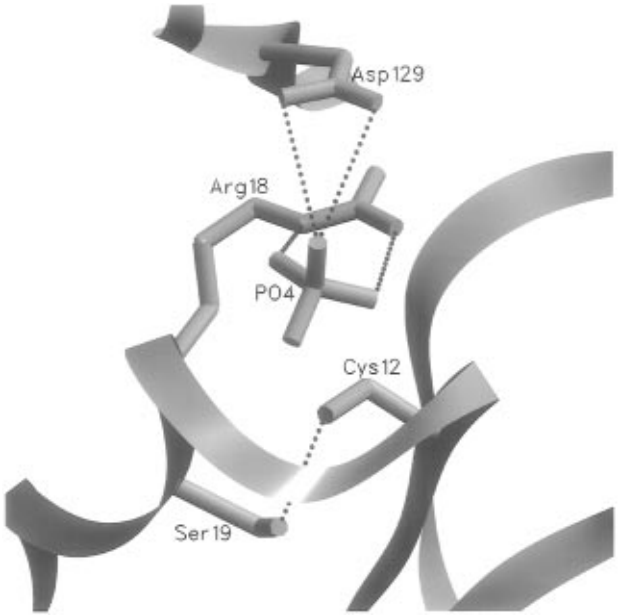


FIGURE 1: Active site features of the bovine low-molecular mass phosphatase (Zhang et al., 1994). The nucleophile Cys12 and the general acid Asp129 are positioned on opposite sides of the phosphate. Arg18 forms bidentate hydrogen bonds with the phosphate, and Ser19 makes a hydrogen bond with the sulfur of Cys12.

residues is observed in the oxyanion (e.g., tungstate, phosphate, or sulfate)-bound structures of the *Yersinia* PTPase, human PTP1B, human dual-specificity phosphatase VHR, and the bovine low-molecular mass phosphatase (Fauman & Saper, 1996).

Recently heavy-atom kinetic isotope effects were used to study the mechanisms of the *Yersinia* PTPase and rat PTP1 (Hengge et al., 1995) and the human dual-specificity phosphatase VHR (VH1-related) (Hengge et al., 1996). These three enzymes were found to hydrolyze *p*NPP by essentially identical, highly dissociative transition states, similar to the uncatalyzed aqueous reaction. Structural comparisons reveal that the VHR structure defines a highly conserved protein core for the dual-specificity phosphatases and PTPases, while the structures of low-molecular mass phosphatases display similar secondary structure elements, albeit with distinct topologies (Fauman & Saper, 1996). The low-molecular mass phosphatases and PTPases most likely represent examples of convergent evolution.

We report here the results of an isotope effect study with a member of the low-molecular mass tyrosine phosphatase family, Stp1, from the fisson yeast (*Schizosaccharomyces pombe*). Genetic screening indicates that Stp1 can act as a multicopy suppressor of the cell cycle regulator cdc25 temperature-sensitive mutants (Mondesert et al., 1994). Figure 2 shows the isotope effects which were measured in the *p*NPP substrate. This study examines the hydrolysis of *p*NPP by the native Stp1, the general acid mutants D128N, D128A, and D128E, and the mutant S18A. These studies were carried out to compare the mechanistic details of the catalysis of Stp1 with those of other protein phosphatases, and with uncatalyzed phopshoryl transfer.

The enzymatic hydrolysis of phosphate monoesters typically occurs millions of times faster than the uncatalyzed reaction in solution. There is considerable interest in the molecular details of this catalysis, and in whether the mechanisms followed and the transition states differ from those of the uncatalyzed reactions. Phosphate monoesters in solution are hydrolyzed via a concerted S_N2 mechanism (A_ND_N in the IUPAC nomenclature). The transition state is highly dissociative with bond cleavage to the leaving group nearly complete and with a very small degree of bond formation to the nucleophile. Dissociative transition states are exhibited both in the reactions of dianionic species, where the leaving group departs as the anion, and in reactions of monoanionic species where the leaving group is protonated in the transition state (Benkovic & Schray, 1978; Thatcher & Kluger, 1989). Enzymatic phosphoryl transfers of phosphate monoesters which have been studied have generally been found to have a similar mechanism (Weiss & Cleland, 1989; Jones et al., 1991; Admiraal & Herschlag, 1995; Hollfelder & Herschlag, 1995; Hengge et al., 1995, 1996). However, an alkaline phosphatase mutant in which the nucleophilic Ser was mutated to Cys has been reported to proceed with a more associative transition state with less bond cleavage to the leaving group (Han & Coleman, 1995). Also, the dephosphorylation of the phosphocysteine intermediate in the low-molecular mass protein-tyrosine phosphatase Stp1 reaction has been found to become less dissociative in a mutant where hydrogen bonding interactions with the Cys leaving group are removed in a site-directed mutant (Zhao & Zhang, 1996). It has been pointed out that an enzymatic phosphoryl transfer reaction could in theory assume a more associative transition state if catalytic groups stabilize the increased negative charge on the nonbridge oxygen atoms that would result from such a mechanism (Knowles, 1980; Hasset et al., 1982).

MATERIALS AND METHODS

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

Site-directed mutagenesis was carried out using the MutaGene *in vitro* Mutagenesis kit from Bio-Rad. The plasmid

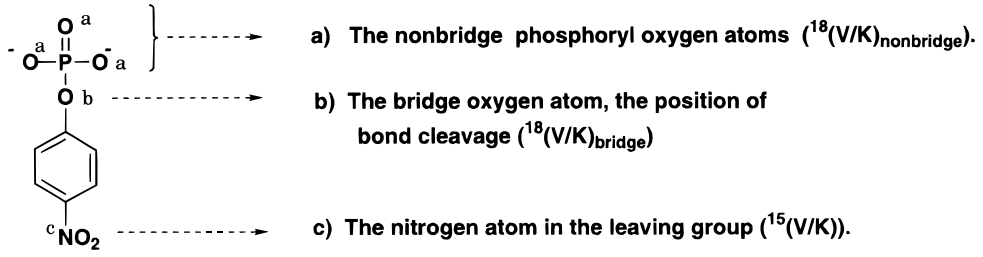


FIGURE 2: *p*-Nitrophenyl phosphate substrate showing the positions where isotope effects were measured.

pUC118-Stp1 (Zhang et al., 1995a) encoding the yeast low-molecular mass tyrosine phosphatase, Stp1 (small tyrosine phosphatase), was used to make single-stranded DNA for site-directed mutagenesis. Site-directed mutants, S18A, D128A, D128N, and D128E, of Stp1 were prepared as described previously (Wu & Zhang, 1996; Zhao & Zhang, 1996). The wild type and mutant Stp1 phosphatases were expressed in *E. coli* strain BL21 (DE3) and purified to homogeneity as described (Zhang et al., 1995a; Wu & Zhang, 1996; Zhao & Zhang, 1996).

Steady State Kinetics. The Stp1 phosphatase activity was assayed at 30 °C in a reaction mixture (0.2 mL) containing appropriate concentrations of *p*NPP as substrate. Buffers used were as follows: pH 4.0−5.5, 100 mM acetate; pH 5.5−6.5, 50 mM succinate; pH 6.6−7.3, 50 mM 3,3-dimethylglutarate; and pH 7.5−9.0, 50 mM Tris. All of the buffer systems contained 1 mM EDTA, and the ionic strengths of the solutions were kept at 0.15 M using NaCl. The reaction was initiated by addition of enzyme and quenched after 2−3 min by addition of 1 mL of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18 000 M^{-1} cm^{-1}. The pH dependence of k_{cat}/K_m data was fitted to eq 1 using a nonlinear least-squares regression program (KaleidaGraph, Synergy Software). In eq 1, (k_{cat}/K_m)^{lim} is the pH-independent second-order rate constant, K_{S2}, the second ionization constant of the substrate, and K_{E1} and K_{E2} the ionization constants of the free enzyme.

$$\frac{k_{cat}}{K_m} = \frac{\left(\frac{k_{cat}}{K_m}\right)^{lim}}{\left(1+\frac{H}{K_{S2}}\right)\left(1+\frac{H}{K_{E1}}+\frac{K_{E2}}{H}\right)} \quad (1)$$

The inhibition constant for *p*-nitrophenol was determined for Stp1 in the following manner. The initial rate at various *p*NPP concentrations was measured by following the production of inorganic phosphate (Zhang, 1995). The inhibition constant and inhibition pattern were evaluated using a direct curve-fitting program KINETASYST (IntelliKinetics, State College, PA).

General Method for Isotope Effect Measurements. The isotope effects in this study were measured by the competitive method, using an isotope ratio mass spectrometer. The ^{18}O isotope effects were measured by the remote label method (O'Leary & Marlier, 1979). In this technique, substrate is synthesized with labels at two positions, one at the site of chemical interest and the other, which serves as the remote label, at a position which lends itself to facile isolation and isotopic measurement. This doubly labeled material is mixed with substrate containing only the natural abundance of ^{18}O in the position of interest, but depleted material in the remote label position (see Figure 3). The mixing ratio is such that the natural abundance of ^{15}N is restored in the remote label position. When this mixture is used in an experiment, the observed isotope effect is the product of that in the position of interest and that in the remote label position. The isotope effect in the latter position is determined with normal substrate containing only the natural abundance of isotopes in all positions, and the ratio of the two observed isotope effects is the desired one in the position of interest. With *p*NPP, the nitrogen atom serves as a convenient remote label which is easily isolated as molecular nitrogen, as previously described (Hengge et al., 1994).

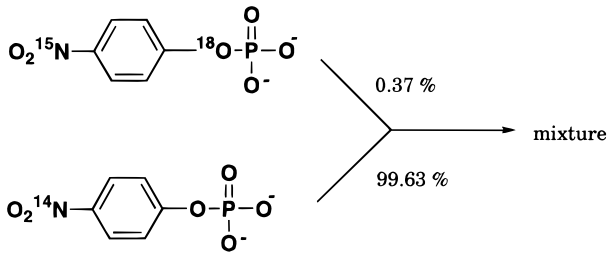


FIGURE 3: Diagrammatic representation of the preparation of the mixture used to measure ^{18}O isotope effects in *p*NPP [in this case $^{18}(V/K)_{bridge}$] using the remote label method, where the nitrogen atom in the nitro group serves as the remote label.

Kinetic Isotope Effect Measurements. The isotope effect experiments performed at pH 5.0, 6.0, and 8.0 were performed at 37 °C in buffered solutions (0.1 M) of acetate, Bis-Tris, and Tris, respectively, with 1 mM EDTA. For measurement of each isotope effect, three reactions were started with approximately 100 μmol of *p*NPP substrate in each. After the solutions were equilibrated at 37 °C, sufficient enzyme was added in order to give a half-life for conversion to product of about 3 h with native enzyme to a maximum of 36 h with the slower mutants. Control experiments determined that the uncatalyzed hydrolysis of *p*NPP was negligible (less than 0.5%) over the longest reaction time used. The set of three reactions were allowed to proceed to varying fractions of reaction, from around 20 to 70%. Reaction progress was followed by monitoring the appearance of *p*-nitrophenol from the absorbance at 400 nm of an aliquot of the reaction mixture added to 0.2 N NaOH. When approximately the desired fraction of reaction was reached, an aliquot was removed for determination of the exact fraction of reaction and then reaction progress was stopped by chilling the flasks in ice. This was followed shortly by titration to pH 5−6 and extraction of the *p*-nitrophenol with diethyl ether (3 × 25 mL), which leaves unreacted *p*NPP in the aqueous layer. After the ether extracts were dried over magnesium sulfate, the ether was removed by rotary evaporation. The aliquot which was removed prior to ether extraction was diluted into Tris buffer at pH 9 and used to determine the fraction of reaction by assaying for free *p*-nitrophenol at 400 nm, treating with alkaline phosphatase overnight, and re-assaying at 400 nm.

The unreacted *p*NPP substrate in the aqueous layer was completely hydrolyzed by treatment with alkaline phosphatase (3 units) at pH 9 in Tris buffer which was 1 mM in both Zn and Mg. The *p*-nitrophenol thus produced was isolated by acidification to pH 6 and ether extraction, as for the initial product. The *p*-nitrophenol samples from both product and residual substrate at partial reaction were further purified by sublimation and then combusted to produce nitrogen gas which was isolated and analyzed by isotope ratio mass spectrometry as previously described (Hengge et al., 1994).

Isotope effects were calculated from the isotopic ratio of nitrogen in the *p*-nitrophenol product at partial reaction (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equation 2 was used to calculate the observed isotope effect

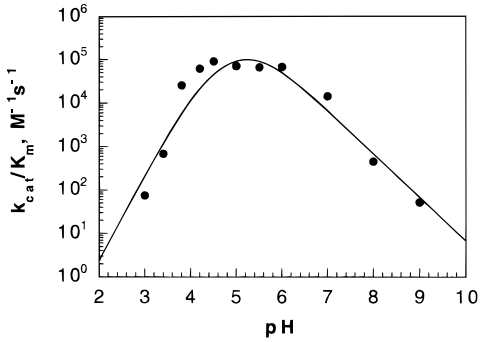


FIGURE 4: pH−k_{cat}/K_m profile for the wild type Stp1-catalyzed hydrolysis of *p*NPP. The line that is drawn through the experimental data is based on the nonlinear least-squares fit of the data to eq 1. The pK_{S2} for pNPP substrate was assigned as 5.0. pK_{E1} = 4.09 ± 0.23, and pK_{E2} = 5.39 ± 0.19. The slope for the ascending limb is +2 and for the descending limb is −1.

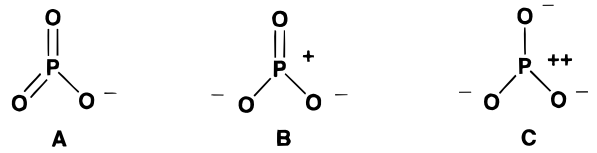


FIGURE 5: Possible resonance structures for the metaphosphate ion.

from product and starting material at fraction of reaction *f*, and eq 3 was used to calculate the observed isotope effect from residual substrate and starting material. The observed isotope effects from experiments to determine ^{18}O isotope effects were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material (Caldwell et al., 1991).

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

$$\text{isotope effect} = \log(1-f)/\log[(1-f)(R_s/R_o)] \quad (3)$$

The isotopic ratio of nitrogen in the starting material, R_o, was determined both by direct combustion of samples of *p*NPP substrate and, as a control, by completely hydrolyzing samples of *p*NPP and analyzing the liberated nitrophenol following isolation 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.

RESULTS

Figure 4 shows the k_{cat}/K_m−pH profile for the wild type Stp1 enzyme. The second ionization constant pK_{S2} for the *p*NPP substrate used in the fit was 5.0; the enzymatic pK_a values from the computer fit to the data are 4.09 ± 0.23 and 5.38 ± 0.19. The slope for the acid limb is +2, while that for the basic limb is −1. This pH−rate behavior is very similar to that observed for other members of this family of phosphatases (Zhang et al., 1994a; Zhang, 1995; Denu et al., 1995) and indicates that substrate is bound as the dianion and attack is by the negatively charged thiolate, with general acid catalysis (Zhang, 1997).

Table 1 lists the isotope effects and their standard errors for the reactions of *p*NPP with native Stp1 and the mutant enzymes. Each number is the average of at least six independent determinations. In each case, the isotope effect calculated from the isotope ratios of residual substrate and starting material was within experimental error of that calculated from the ratios of product and starting material, and these were averaged together to give the results in Table 1. The largest isotope effect, $^{18}(V/K)_{bridge}$, was determined for the native Stp1 both near the pH optimum of 5.0 and also at 6.0 and 8.0, where chemistry is slower and thus potentially more rate-limiting in the event a nonchemical step is partially rate-limiting at the optimum pH. Isotope effects with the mutant enzymes were all measured at pH 6.0.

Table 1: Kinetic Isotope Effects on Reactions of Stp1 and mutants with *p*NPP

Stp1 Reactions[a]

pH	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$[b]
native Stp1 (pH 5)		1.0171 ± 0.0006	
native Stp1 (pH 6)	1.0007 ± 0.0001	1.0160 ± 0.0005	1.0018 ± 0.0003
native Stp1 (pH 8)		1.0161 ± 0.0005	
D128N (pH 6)	1.0034 ± 0.0003	1.0282 ± 0.0012	1.0024 ± 0.0005
D128A (pH 6)	1.0030 ± 0.0005	1.0297 ± 0.0009	1.0010 ± 0.0006
D128E (pH 6)	1.0006 ± 0.0002	1.0166 ± 0.0010	1.0013 ± 0.0003
S18A (pH 6)	1.0010 ± 0.0002	1.0172 ± 0.0013	1.0024 ± 0.0005

Solution Reactions[c]

	^{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

[a] Reaction conditions: 0.1 M buffer and 1 mM EDTA at 37 °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 aqueous hydrolysis reactions of *p*NPP from Hengge et al. (1994).

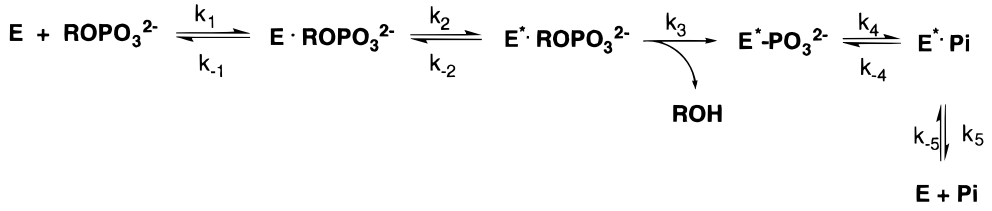
The values for both of the ^{18}O isotope effects $^{18}(V/K)_{bridge}$ and $^{18}(V/K)_{nonbridge}$ in the table have been corrected for the ^{15}N effects. Because the enzymatic substrate is the dianion of *p*NPP (Zhang et al., 1994a; Zhang, 1995; Denu et al., 1995; Y. Zhao and Z.-Y. Zhang, unpublished results), the values for $^{18}(V/K)_{nonbridge}$ have also been corrected for the equilibrium ^{18}O isotope effect on protonation, as previously described (Hengge et al., 1994). The values for $^{18}(V/K)_{nonbridge}$ given in the table are the effects resulting from isotopic labeling of all three nonbridge oxygen atoms.

For the sake of comparison with the uncatalyzed reactions of *p*NPP, the isotope effects determined in previous work (Hengge et al., 1994) for the aqueous hydrolysis of the *p*NPP monoanion and dianion are given at the bottom of Table 1. These reactions were carried out at 95 °C; the values reported in Table 1 have been corrected to 35 °C using the approximation ln(effect at 37 °C) = (368 K/310 K) ln(effect at 95 °C).

DISCUSSION

Kinetic isotope effects can be used to characterize phosphoryl transfer reactions in detail, in particular yielding information about transition state structure. The primary isotope effect $^{18}(V/K)_{bridge}$ (Figure 2) gives a measure of the degree of cleavage of the P−O bond in the transition state. The $^{15}(V/K)$ isotope effect is sensitive to the amount of negative charge borne by the leaving group *p*-nitrophenol and thus gives information as to whether the leaving group departs as the anion or if protonation of the leaving group has neutralized all or part of the negative charge resulting from bond rupture (Hengge & Cleland, 1990). The secondary isotope effect $^{18}(V/K)_{nonbridge}$ reveals whether this group resembles metaphosphate as in a dissociative transition state or if it has a phosphorane-like structure expected from an

Scheme 1[a]



[a] In this study, R = *p*-nitrophenyl.

associative process. Because only isotope effects on rate-limiting steps are experimentally observable, the magnitude of the isotope effects also gives a measure of the degree to which the chemical step is rate-limiting in the enzymatic reaction.

Scheme 1 shows the overall mechanism of the PTPase reaction. Reaction proceeds through a sequence involving binding of substrate followed by a binding-induced conformational change which brings the general acid close to the scissile oxygen of the substrate (Stuckey et al., 1994; Jia et al., 1995). This ligand-induced loop closure is likely to be operative for the entire PTPase family, although this has not been confirmed for the dual-specificity phosphatases and the low-molecular mass phosphatases, since only the ligand-bound structures have been determined (Su et al., 1994; Zhang et al., 1994; Yuvaniyama et al., 1996). Substrate is then cleaved with phosphoryl transfer to the nucleophilic Cys residue. Subsequent base-catalyzed reaction with water cleaves the phosphoenzyme intermediate, and release of phosphate completes the catalytic cycle. The rate-limiting step for the overall reaction of Stp1 is the second chemical step, k_4 (Zhang et al., 1995a; Wu & Zhang, 1996). The transition state for this step has been examined by using linear free energy relationships (Zhao & Zhang, 1996). The isotope effects in the present study were determined by the competitive method and are thus those on *V*/*K* (Northrop, 1982). Therefore, they are sensitive only to steps up to and including the first irreversible step in the mechanism, which is represented in Scheme 1 as the phosphoryl transfer step k_3 accompanied by release of *p*-nitrophenol. The justifications for representing this step as irreversible are the high K_i (140 mM) for *p*-nitrophenol (a product and a noncompetitive inhibitor), which indicates that it binds poorly to the active site and should be released quickly, and also the poor nucleophilicity of neutral *p*-nitrophenol.

These isotope effects allow the selective examination of the first chemical step in the mechanism, to the degree that it is rate-limiting on *V*/*K*. The interpretation of the data thus requires knowledge of the extent to which the phosphoryl transfer step is rate-limiting. To the extent that either substrate binding or the associated conformational change is not in rapid equilibrium, and thus partially limits the rate, the magnitude of the isotope effects on the chemical step will be suppressed or completely abolished (the latter resulting in observed isotope effects of unity).

When only one step is isotopically sensitive in an enzymatic reaction, the isotope effect on *V*/*K* is described by eq 4 (Cleland, 1987). In this equation, *(*V*/*K*) represents the isotope effect on *V*/*K* and **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).

$$*(V/K) = [*k + c_f + c_r(*K_{eq})]/(1 + c_f + c_r) \quad (4)$$

There will be no reverse commitment if the phosphoryl transfer step k_3 is irreversible, which is a fair assumption with the substrate *p*NPP as discussed above. If it is the only isotope-sensitive step, then the expression for the isotope effect can be reduced to eq 5.

$$*(V/K) = (*k_3 + c_f)/(1 + c_f) \quad (5)$$

where $c_f = (k_3/k_{-2})(1 + k_2/k_{-1})$.

It is uncommon for the chemical step to be completely rate-limiting in the reaction of an enzyme with its natural substrate, as the tight binding typically found with these substrates limits the reversibility of the binding step and thus introduces a commitment toward catalysis (Cook, 1991). 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. The natural substrates of PTPases are phosphorylated proteins with substantial binding interactions coming from the peptide residues adjacent to the phosphorylated residue (Zhang et al., 1994b; Jia et al., 1995). This fact gives us a reasonable expectation that the small, alternate substrate *p*NPP will not be a "sticky" substrate and the ratio k_2/k_{-1} will be small. The ratio k_3/k_{-2} reflects the partitioning of the enzyme−substrate complex following the likely conformational change bringing the peptide loop bearing the catalytic general acid near the active site. 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. Isotope effect studies with other members of the PTPase superfamily indicate that with the substrate *p*NPP this conformational change is rapidly reversible and does not induce a commitment toward catalysis (Hengge et al., 1995, 1996).

The best experimental test of whether chemistry is rate-limiting is to compare the magnitudes of the isotope effects on an enzymatic reaction with those for a similar reaction performed in solution, since the uncatalyzed reaction will not be subject to possible commitments from nonchemical steps. Another useful procedure is to compare the magnitudes of the enzymatic isotope effects measured at the pH optimum and at a pH well away from the optimum, where the chemical step will be slower. If chemistry is only partially rate-limiting at the pH optimum but more so at a nonoptimal pH, the magnitudes of the isotope effects will be larger in the latter case. Only if commitment factors are negligible will the full intrinsic isotope effects on the chemical step be expressed at the pH optimum, and their magnitudes will in that case not increase as a result of changes in the pH of reaction.

In phosphoryl transfer reactions of *p*NPP, the largest isotope effect is the primary effect at the position of bond cleavage, $^{18}(V/K)_{bridge}$. The large magnitude of this isotope effect makes it the one most sensitive to the presence of a commitment, and therefore, its magnitude was determined at the pH optimum of 5.0, as well as at pH 6.0 and 8.0. The analogous aqueous hydrolysis reaction with respect to this isotope effect site is that of the monoanion, where proton transfer to the bridge oxygen occurs with bond cleavage, as is the case in the enzymatic reaction. The value of $^{18}(V/K)_{bridge}$ is large even at the pH optimum and does not increase as the reaction is moved to pH 8.0. The measured effect of about 1.0161 is larger than that of the monoanion in solution and is very close to the corresponding effects previously seen with the *Yersinia* PTPase and rat PTP1 (Hengge et al., 1995). We therefore conclude that the bond cleavage step is rate-limiting for *V*/*K* at the pH optimum and that the isotope effects measured are the intrinsic ones for cleavage of the P−O bond. Since the mutant enzymes have slower rates than the native Stp1, this conclusion should be valid for data obtained with the mutants as well. These data also indicate that, if there is a conformational change which swings the peptide loop bearing the general acid Asp128 into the active site, it must also be rapidly reversible in the reactions studied.

Transition State Structures

Native Stp1. The ^{15}N isotope effect is sensitive to the presence of negative charge on the departing leaving group. The magnitude of this effect varies in a systematic fashion as expected in reactions of monoesters, diesters, and triesters of *p*-nitrophenol (Hengge & Cleland, 1990). This isotope effect is largest in the aqueous hydrolysis of the dianion where essentially a full negative charge is developed on the leaving group in the transition state. Its magnitude is smaller in diester reactions and is smaller still in reactions of triesters. In this series, the transition states become more associative and transition state bond cleavage becomes less advanced. The ^{15}N effect nearly disappears in the aqueous hydrolysis of the monoanion, where proton transfer to the leaving group has nearly completely neutralized the negative charge arising from nearly complete P−O bond cleavage in the late transition state for this reaction. The small but experimentally significant value of 1.0007 for $^{15}(V/K)$ with the native Stp1 is about $^1/_5$ as large as that seen in the aqueous hydrolysis of the dianion. This result indicates that proton transfer to the leaving group lags behind P−O bond cleavage, resulting in the leaving group bearing a small amount of negative charge in the transition state. This contrasts with the results found in the other protein phosphatases examined to date which all have exhibited $^{15}(V/K)$ values of unity within experimental error (Hengge et al., 1995, 1996), indicating that in those cases charge neutralization due to proton transfer is complete in the transition state.

Another difference found in the native Stp1 is the normal $^{18}(V/K)_{nonbridge}$ effect of 1.0018 which contrasts with the corresponding measurements with the other protein phosphatases. 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 (typically from 1.003 to 1.006) for diesters of *p*-nitrophenol where the transition state is somewhat associative, and values that are larger and normal (1.006−1.025) for triesters where the reaction is more associative (Caldwell et al., 1991). Thus, the $^{18}(V/K)_{nonbridge}$ isotope effect is a predictable and valuable tool in measuring the dissociative versus associative character of a transferring phosphoryl group in the transition state.

Values of $^{18}(V/K)_{nonbridge}$ obtained for the other protein phosphatases, the *Yersinia* PTPase, PTP1, and VHR, have all been unity or slightly inverse, as is the case for the solution hydrolysis of the dianion. This is consistent with the known dissociative nature of this reaction and the metaphosphate-like nature of the phosphoryl group in the transition state (Hengge et al., 1995, 1996). Computational results indicate that metaphosphate is best described as a resonance hybrid of the three structures in Figure 5, the main contributors being forms B and C (Rajca et al., 1987; Horn & Alrichs, 1990). This picture of metaphosphate predicts minimal values for $^{18}(V/K)_{nonbridge}$ in contrast to the classical picture of this species as represented by structure A, which would predict significant inverse values for this isotope effect due to the increased P−O bond order in the nonbridge oxygen atoms relative to the substrate. While the *Yersinia* PTPase, PTP1, and VHR do not differ measurably from the aqueous reaction with regard to the structure of the transferring phosphoryl group, the small normal $^{18}(V/K)_{nonbridge}$ Stp1 effect is indicative of a small loss of nonbridge P−O bond order in the transition state. The effect measured with the Stp1 reaction suggests a phosphoryl group that is intermediate in structure between the metaphosphate-like monoester reaction and the somewhat associative structure found in diester reactions. This result suggests a greater role for the nucleophilic Cys anion in this reaction than the minimal nucleophilic interaction typical of monoester reactions.

With the previously studied protein phosphatases, mutation of the general acid to asparagine or alanine gave rise to small normal $^{18}(V/K)_{nonbridge}$ isotope effects similar in magnitude to those found in the present study with native Stp1, in contrast to the unity or slightly inverse value with the other native enzymes (Hengge et al., 1995, 1996). This was interpreted either as being due to increased nucleophilic interactions by the Cys anion in the transition state or as a result of an alteration in the resonance contributions of metaphosphate-like phosphoryl group as a result of the increased negative charge in the transition state (Hengge et al., 1996). Loss of the general acid means that the negatively charged phosphoryl group in the transition state is sandwiched between the anionic nucleophile and the anionic leaving group, and it was proposed that this may enhance the contributions of resonance structure C and result in the small normal value observed for $^{18}(V/K)_{nonbridge}$. However, in the native Stp1 reaction, the leaving group bears only a small fractional negative charge as indicated by the $^{15}(V/K)$ isotope effect, which should not give rise to measurable differences in electronic structure of the phosphoryl group. Thus, the loss of nonbridge P−O bond order indicated by the $^{18}(V/K)_{nonbridge}$ effect in the native Stp1 is much more likely to be due to a small degree of nucleophilic participation by the Cys nucleophile, less than that exhibited in diester reactions but more than is typical of monoester reactions. In the *Yersinia* PTPase, in PTP1, and in VHR, the $^{18}(V/K)_{nonbridge}$ isotope effects change from unity or slightly inverse for the wild type enzymes to small normal effects ranging from 1.0018 to 1.0022 in the D to N mutants (Hengge et al., 1995, 1996). This change is presumably the result of an increase

in nucleophilic participation by the thiolate, which can be rationalized by the need for a greater "push" to expel the leaving group in the absence of general acid assistance. Thus, both the small $^{15}(V/K)$ isotope effect and the normal $^{18}(V/K)_{nonbridge}$ effects point to a transition state for the wild type Stp1 reaction in which slightly more nucleophilic involvement is coupled with incomplete general acid assistance.

The $^{18}(V/K)_{bridge}$ isotope effect of 1.6% found for the native Stp1 is similar to that found with the other protein phosphatases and is slightly larger than that found in the aqueous monoanion reaction. This effect is smaller than in the aqueous hydrolysis of the dianion because the cleavage of the P−O bond is partially compensated for by the formation of the O−H bond in the transition state. The large magnitude of $^{18}(V/K)_{bridge}$ with native Stp1, where proton transfer to the leaving group is significant though incomplete, indicates that the degree of transition state bond cleavage is large.

The overall picture of the transition state with the native enzyme is one which is highly dissociative with respect to leaving group departure but which exhibits isotopically measurable nucleophilic interaction between the Cys anion and the phosphoryl group. This latter characteristic distinguishes it from the *Yersinia* PTPase, PTP1, and VHR, and from the aqueous hydrolysis of *p*NPP.

A recent mechanistic proposal based on computational results asserts either that PTPases react via a proton transfer from Cys to the phosphoryl group during reaction or that the substrate binds as the monoanion (Hansson et al., 1997). These workers rule out binding of the dianion to enzyme with the Cys deprotonated on the basis of the charge repulsion that would result from bringing together a singly and a doubly negatively charged group. However, pH−rate profiles and pH-dependent inactivation data for all PTPases examined to date indicate that the active form of the substrate is the dianion and that the active site Cys exists as a thiolate anion at physiological pH [see Zhang (1997) for a review]. This is probably facilitated by the partial charge neutralization conferred by the conserved cationic Arg and backbone amide H bonds in the phosphate binding loop. With regard to proton transfer during catalysis, the $^{18}(V/K)_{nonbridge}$ isotope effects for Stp1 and all other PTPases are inconsistent with proton transfer to the phosphoryl group; such protonation would result in large inverse effects (Weiss & Cleland, 1986) which are not observed.

General Acid Mutants D128N and D128A. With the loss of the general acid, the leaving group departs as the *p*-nitrophenolate anion, which is reflected in the sizable $^{15}(V/K)$ isotope effect that arises from the delocalization of charge into the aromatic ring. In addition, the value of $^{18}(V/K)_{bridge}$ is significantly increased over that of the reaction of the native enzyme. Protonation of *p*-nitrophenolate gives rise to an inverse ^{18}O isotope effect, and the loss of this inverse contribution to $^{18}(V/K)_{bridge}$ results in a larger normal isotope effect due to P−O bond cleavage. For this same reason, the bridge ^{18}O isotope effect in the aqueous hydrolysis of the dianion of *p*NPP is larger than that of the monoanion, since in the latter reaction bond cleavage is accompanied by proton transfer. In the D128N mutant, the general acid is replaced by an asparagine which has the potential for stabilization of the *p*-nitrophenolate leaving group through hydrogen bonding, and in D128A by a residue which should not be capable of any stabilizing interaction. Kinetic data indicate that the rate of the phosphoryl transfer from *p*NPP is reduced 11900-fold in D128N and 7480-fold in D128A (Wu & Zhang, 1996). The isotope effects at each position are identical for these two mutants within experimental error. This rules out any significant hydrogen bonding contribution by the asparagine in D128N, as the data indicate that an identical degree of charge is borne on the leaving group for these two mutants. The data indicate that the differing rate reductions of these mutants are not accompanied by any alteration in transition state structure.

The $^{18}(V/K)_{nonbridge}$ effect for D128N is slightly larger (more normal) than that for the wild type enzyme. This trend is identical to that seen with other PTPases and is consistent with the need for greater nucleophilic involvement in the absence of general acid assistance. The D128A mutant however exhibits a smaller $^{18}(V/K)_{nonbridge}$ effect than D128N and is also smaller than that in the wild type reaction. With the *Yersinia* PTPase, D356A also exhibited a smaller value for $^{18}(V/K)_{nonbridge}$ than D356N. We have no explanation for this observation; there is no evident structure−reactivity reason for a difference in transition state structure to exist between these mutants. The alanine mutation is a less conservative change, and it is possible that a structural alteration in the catalytic complex results which alters substrate−enzyme interactions, though we have no evidence of such a change.

The wild type Stp1 exists predominantly in the general acid D128-deprotonated form at pH 8 (Figure 4). Thus, one might have expected that substrate turnover by this deprotonated form of the enzyme would result in a similar transition state structure and therefore isotope effects similar to those observed in the general acid mutants D128N and D128A. However, the fact that the isotope effects for the wild type enzyme at pH 8.0 are the same as those at pH 5.0 and 6.0 implies that the activity seen at pH 8.0 is due to the remaining amount of correctly protonated enzyme present at this pH and that turnover by the deprotonated enzyme is not competitive. This is supported by the pH−rate data. The observation of a slope of −1 in this region on the basic side of the pH−rate profile for wild type enzyme indicates that activity drops off in direct proportion to the diminishing proportion of correctly protonated enzyme present. If the deprotonated form had activity at competitive levels, this would result in a reduced slope in this region of the pH profile. This is further supported by the much smaller k_{cat}/K_m values exhibited by general acid mutants compared with those of the wild type enzyme at pH 8. At pH 8.0, k_{cat}/K_m for wild type Stp1 is 457 s^{-1} M^{-1}, compared with 70 for D128A and 26 for D128N. The fact that general acid catalysis remains effective in the native enzyme at pH 8, which is above the pK_a of *p*-nitrophenol, is not surprising since the leaving group is protonated by the enzymatic general acid, not by solvent buffer, and the loop bearing the general acid closes down on the active site during catalysis which likely shields the substrate from solvent.

D128E Mutant. This substitution maintains an acidic residue at this position, but the extra methylene group of Glu apparently results in some positioning-induced impairment in protonation of the leaving group, as the substitution reduces the rate of phosphoryl transfer by 17-fold relative to that of the native Stp1 (Wu & Zhang, 1996). We were interested in seeing if there was any change in the degree of proton transfer in the transition state that may have arisen from an alternative positioning of the carboxylic acid group

during catalysis. The general acid rests on a flexible loop which is apparently quite mobile even after substrate binding, as the isotope effect data show that this conformational change is rapidly reversible. The identity of the isotope effect data within experimental error at all three positions between the D128E mutant and the native enzyme means that an identical transition state is attained in both reactions. This is also consistent with the observation that the native Stp1 and the D128E mutant displayed effectively identical β_{lg} values for the phosphorylation step (the *V*/*K* term) (Wu & Zhang, 1996). The 17-fold reduction in the rate is then not the result of reaction having to proceed through a less favorable transition state as is the case with the other general acid mutants but rather reflects an impairment in reaching the geometry necessary to achieve the same transition state that is operative in the native enzyme.

S18A Mutant. The low-molecular mass phosphatases, as well as the PTPases and the dual-specificity phosphatases, contain a conserved Ser or Thr residue in the active site sequence (H/V)C(X)$_5$R(S/T) which is within hydrogen bonding distance of the active site Cys nucleophile (Zhang et al., 1995b) (see Figure 1). Site-directed mutagenesis and kinetic experiments indicate that this hydroxyl group of Ser 18 plays an important role in hydrolysis of the phosphoenzyme intermediate (Zhao & Zhang, 1996). The S18A mutant exhibits only about a 4-fold reduction in the rate of phosphoryl transfer from *p*NPP, but a much larger reduction in the rate of the second chemical step. Linear free energy relationships indicate that in the S18A mutant the transition state for the cleavage of the phosphoenzyme intermediate exhibits more nucleophilic involvement than in the native enzyme (Zhao & Zhang, 1996).

This mutation can potentially affect the transition state of the first phosphoryl transfer step as well, since elimination of the hydrogen bonding to the Cys anion will increase its nucleophilicity. The isotope effects measured with S18A on *V*/*K* at each of the three positions are within experimental error of those for the reaction with native enzyme, indicating no alteration in transition state structure for the phosphoryl transfer step from *p*NPP to Cys as a result of the S18A mutation.

CONCLUSIONS

We have shown that the phosphoryl transfer step from *p*NPP is rate-limiting for *V*/*K* in the Stp1 phosphatase, as with other enzymes in this family that have been studied to date. However, the transition state structure for the Stp1 reaction differs by exhibiting a partial negative charge on the leaving group arising from a smaller degree of proton transfer from the general acid, and by exhibiting more nucleophilic participation by the Cys nucleophile. Although protein phosphatases containing the active site sequence (H/V)C(X)$_5$R(S/T) employ the same chemical mechanism for phosphate monoester hydrolysis, the low-molecular mass phosphatases display a topological framework different from that of the PTPases and the dual-specificity phosphatases. It is interesting that measurable differences exist between the transition states of the Stp1-catalyzed reaction and of the *Yersinia* PTPase-, PTP1-, and VHR-catalyzed reaction. Mutation of the general acid from Asp to Glu retards the rate of reaction, but this is not the result of a forced alteration in transition state structure, which is identical to that of the native enzyme. Even with the small amount of nucleophilic participation implied by the $^{18}(V/K)_{nonbridge}$ isotope effects, the transition state is still best described as dissociative in nature with bond cleavage to the leaving group well advanced, as in the solution reaction of phosphate monoesters.

Mutation of the general acid to either Asn or Ala results in a transition state in which the leaving group departs as the anion. These reactions are highly dissociative with respect to leaving group departure but exhibit some nucleophilic involvement. The loss of hydrogen bonding interactions with the Cys nucleophile in the S18A mutant, which affects the transition state structure for breakdown of the phosphoenzyme intermediate, has no measurable affect on the transition state structure for the first step, phosphoryl transfer from substrate to Cys.

REFERENCES

Admiraal, S. J., & Herschlag, D. (1995) *Chem. Biol. 2*, 729−739.

Benkovic, S. J., & Schray, K. J. (1978) The Mechanism of Phosphoryl Transfer, in *Transition States of Biochemical Processes* (Gandour, R. D., Ed.) pp 493−527, Plenum, New York.

Caldwell, S. R., Raushel, F. M., Weiss, P. M., & Cleland, W. W. (1991) *Biochemistry 30*, 7444−7450.

Cleland, W. W. (1987) *Bioorg. Chem. 15*, 283−302.

Cleland, W. W., & Hengge, A. C. (1995) *FASEB J. 9*, 1585−1594.

Cook, P. F. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) p 243, CRC Press, Boca Raton, FL.

Denu, J. M., Zhou, G., Guo, Y., & Dixon, J. E. (1995) *Biochemistry 34*, 3396−3403.

Fauman, E. B., & Saper, M. A. (1996) *Trends Biochem. Sci. 21*, 413−417.

Han, R., & Coleman, J. E. (1995) *Biochemistry 34*, 4238−4245.

Hansson, T., Nordlund, P., & Aqvist, J. (1997) *J. Mol. Biol. 265*, 118−127.

Hasset, A., Blättler, W., & Knowles, J. R. (1982) *Biochemistry 21*, 6335−6340.

Hengge, A. C., & Cleland, W. W. (1990) *J. Am. Chem. Soc. 112*, 7421−7422.

Hengge, A. C., Edens, W. A., & Elsing, H. (1994) *J. Am. Chem. Soc. 116*, 5045−5049.

Hengge, A. C., Sowa, G. A., Wu, L., & Zhang, Z.-Y. (1995) *Biochemistry 34*, 13982−13987.

Hengge, A. C., Denu, J. M., & Dixon, J. E. (1996) *Biochemistry 35*, 7084−7092.

Hollfelder, F., & Herschlag, D. (1995) *Biochemistry 34*, 12255−12264.

Horn, H., & Alhrichs, R. (1990) *J. Am. Chem. Soc. 112*, 2121−2124.

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.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877−919.

Mondesert, O., Moreno, S., & Russell, P. (1994) *J. Biol. Chem. 269*, 27996−27999.

Northrop, D. B. (1977) 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.

Stuckey, J. A., Fauman, E. B., Schubert, H. L., Zhang, Z.-Y., Dixon, J. E., & Saper, M. A. (1994) *Nature 370*, 571−575.

Su, X.-D., Taddei, N., Stefani, M., Ramponi, G., & Nordlund, P. (1994) *Nature 370*, 575−578.
Thatcher, G. R. J., & Kluger, R. (1989) Mechanism and Catalysis of Nucleophilic Substitution in Phosphate Esters, *Adv. Phys. Org. Chem. 25*, 99−265.
Tonks, N. K., & Neel, B. G. (1996) *Cell 87*, 365−368.
Weiss, P. M., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 2759−2761.
Weiss, P. M., & Cleland, W. W. (1989) *J. Am. Chem. Soc. 111*, 1928−1929.
Wu, L., & Zhang, Z.-Y. (1996) *Biochemistry 35*, 5426−5434.
Yuvaniyama, J., Denu, J. M., Dixon, J. E., & Saper, M. A. (1996) *Science 272*, 1328−1331.
Zhang, M., Van Etten, R. L., & Stauffacher, C. V. (1994) *Biochemistry 33*, 11097−11105.
Zhang, Z.-Y. (1995) *J. Biol. Chem. 270*, 11199−11204.
Zhang, Z.-Y. (1997) *Curr. Top. Cell. Regul. 35*, 21−68.
Zhang, Z.-Y., Malachowski, W. P., Van Etten, R. L., & Dixon, J. E. (1994a) *J. Biol. Chem. 269*, 8140−8145.
Zhang, Z.-Y., Maclean, D., McNamara, D. J., Dobrusin, E. M., Sawyer, T. K., & Dixon, J. E. (1994b) *Biochemistry 33*, 2285−2290.
Zhang, Z.-Y., Zhou, G., Denu, J. M., Wu, L., Tang, X., Mondesert, O., Russell, P., Butch, E., & Guan, K.-L. (1995a) *Biochemistry 34*, 10560−10568.
Zhang, Z.-Y., Palfey, B. A., Wu, L., & Zhao, Y. (1995b) *Biochemistry 34*, 16389−16396.
Zhao, Y., & Zhang, Z.-Y. (1996) *Biochemistry 35*, 11797−11804.

BI970364C

Corrections

Properly Oriented Heparin−Decasaccharide-Induced Dimers Are the Biologically Active Form of Basic Fibroblast Growth Factor, by Franklin J. Moy, Michal Safran, Andrew P. Seddon, Doug Kitchen, Peter Böhlen, David Aviezer, Avner Yayon,* and Robert Powers*, Volume 36, Number 16, April 22, 1997, pages 4782−4791.

Page 4782. The complete address for the investigators from the Weizmann Institute was not included in the byline. The correct address should read as follows: Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel.

BI975007G