Does Positive Charge at the Active Sites of Phosphatases Cause a Change in Mechanism? The Effect of the Conserved Arginine on the Transition State for Phosphoryl Transfer in the Protein-Tyrosine Phosphatase from *Yersinia*

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Received July 7, 1999

Abstract: Positive charge is uniformly present in the active sites of all known phosphatases. The postulate that this charge imparts a change to the mechanism and the transition state for phosphoryl transfer was examined by comparing kinetic isotope effects with the substrate *p*-nitrophenyl phosphate for reactions of the native protein tyrosine phosphatase from *Yersinia* with data from mutants in which the conserved arginine residue was mutated to Lys or to Ala. The k_{cat} values for both mutants are about 10^4 less than that of the native enzyme but are still nearly 10^5-fold faster than the uncatalyzed rate. Steady-state kinetic data as well as isotope effects showed that both mutations interfere with functioning of general acid catalysis. To examine the effect of positive charge on the transition state free of this additional effect, double mutants were made in which general acid catalysis was removed by mutation of Asp356 to either Asn or Ala in addition to the mutation to Arg. The k_{cat}/K_m values of D356A and D356N are 300−360-fold higher than those of R409A/D356A and R409A/D356N suggesting that the side chain of Arg409 contributes 3.4−3.5 kcal/mol to transition-state stabilization. Comparisons of the isotope effects for reactions of the double mutants with data from general acid single mutants show that mutation of Arg to either Lys or to Ala does not significantly affect the transition state for phosphoryl transfer. This indicates that this residue functions to stabilize the transition state but does not alter it from its structure in the uncatalyzed reaction.

Protein-tyrosine phosphatases (PTPases) have an essential role in signal transduction, and together with protein-tyrosine kinases control the tyrosine phosphorylation level of proteins in the cell. The *Yersinia* PTPase is one member of this enzyme family, which in common with all members of the PTPase family shares the active site signature motif $C(X_5)R(S/T)$.[1] The PTPases employ a common catalytic strategy in catalyzing a double-displacement mechanism[2] in which the phosphoryl group is first transferred from the substrate to the active site Cys residue (Cys403 in the *Yersinia* PTPase) leading to the formation of a thiophosphoryl enzyme intermediate that is subsequently hydrolyzed by water.[1−4] The invariant Arg residue (Arg409 in the *Yersinia* PTPase) functions in substrate binding and in transition-state stabilization.[1,5,6] The initial phosphoryl transfer step is assisted by general acid catalysis by a conserved Asp (Asp-356 in the *Yersinia* PTPase) which protonates the leaving group.[7] The chemical mechanism for the initial phosphoryl transfer step catalyzed by the *Yersinia* PTPase is shown in Figure 1.

Like all phosphatase enzymes, the PTPases must catalyze a reaction that is very slow in solution. Phosphate monoesters are extremely stable species in solution and are extremely resistant to hydrolysis under physiological conditions.[8,9] There is considerable interest in the means by which phosphatases catalyze this reaction. A central part of this lively debate, and an issue in the understanding of enzymatic catalysis in general, is whether the transition state for an enzymatic reaction is altered from that for the uncatalyzed reaction in solution.

Phosphoryl transfer reactions in solution occur by a range of mechanisms, ranging from dissociative (or loose) to associative (or tight), depending on the level of esterification of the phosphate moiety (Scheme 1). In a dissociative or loose transition-state bond cleavage is predominant, and the sum of the bond orders to the nucleophile and leaving group is less than in the reactants. In an associative or tight transition state the sum of these bond orders increases in the transition state. A corollary is that the net charge on the phosphoryl group which is undergoing transfer must change in opposite ways in the two mechanisms. In a loose transition state the phosphoryl group will undergo a reduction in net negative charge, contrasted with a gain in negative charge in a tight transition state.

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10.1021/ja992361o CCC: $18.00
Published on Web 09/29/1999

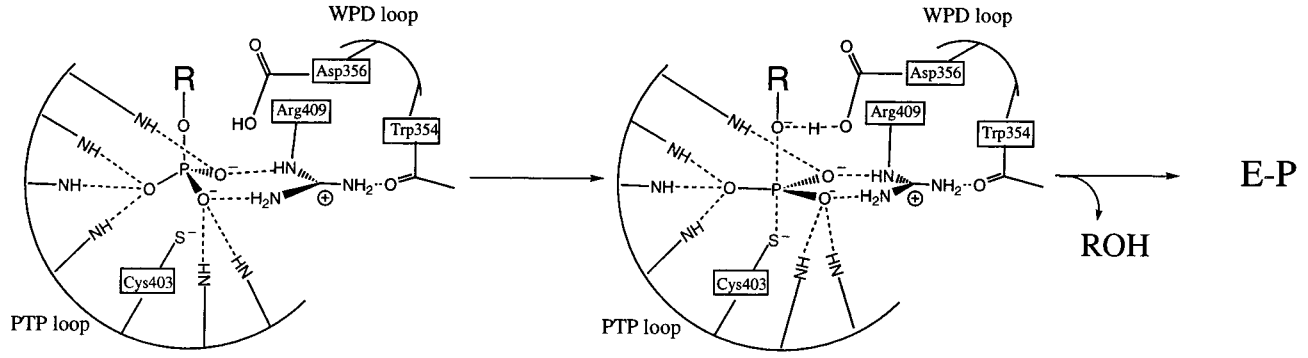
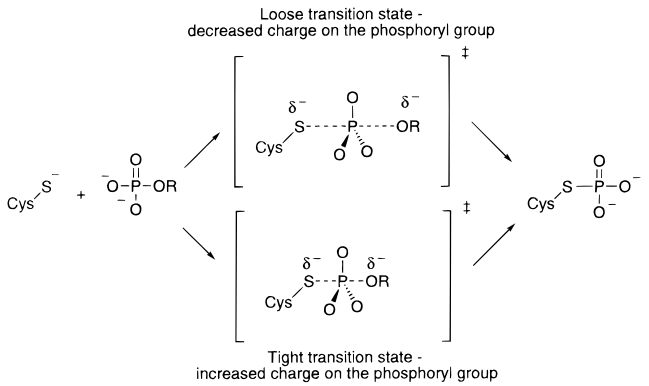


Figure 1. A chemical mechanism for the initial phosphoryl transfer step catalyzed by the *Yersinia* PTPase. R-OH is the phenol; E-P is the thiophosphoryl enzyme intermediate.

Scheme 1



A wealth of experimental data from decades of experiments indicates that phosphate monoesters undergo uncatalyzed reactions via a loose transition state in which the phosphoryl group resembles metaphosphate.[10,11] The dianionic phosphoryl group in the substrate should become essentially monoanionic in such a transition state. Phosphatases utilize differing catalytic strategies and functional groups, and comparisons of their structures show that the only similarity at the active site is the presence of positive charge.[12] In the PTPases this charge is in the form of a conserved arginine residue, Arg409 in the *Yersinia* enzyme. The difficulties faced by an enzyme in stabilizing a dissociative transition state have been pointed out; the presence of positive charge in the enzyme active site might be expected to favor development of *increased* negative charge in the transition state, not the decrease that would occur in the dissociative process.[13] X-ray structures of a number of phosphoryl transfer enzymes with transition-state analogues have been interpreted to favor a transition state that is much more associative than in the uncatalyzed reaction.[14−19] It has been proposed that the presence of positively charged arginine residues around a transferred phosphoryl group demands an associative transition state for enzymatic phosphoryl transfer.[20]

We have previously used heavy atom isotope effects to characterize the transition states of the *Yersinia* PTPase-catalyzed reaction of *p*-nitrophenyl phosphate (*p*NPP) with the native enzyme and general acid mutants. In the present study we have sought to examine the effect of mutations of the conserved arginine residue 409 to lysine or to alanine on the structure of the transition state of the phosphoryl transfer reaction with the substrate *p*NPP. In the lysine mutant a cationic residue is still present, but one which can donate fewer hydrogen bonds to the phosphoryl group than the arginine residue in the native enzyme. In the alanine mutant both the positive charge and all hydrogen bonds between this residue and the substrate are removed. Although significantly reduced in activity, both mutants are capable of catalyzing the phosphoryl transfer reaction at a rate well above the rate of background hydrolysis. Mutation of Arg409 was found to adversely affect a conformational change that enables general acid catalysis. To separate this indirect effect from the direct effect of the cationic Arg409 residue on the nature of the transition state, double mutants were prepared in which the general acid was also mutated, to either Asn or Ala. A comparison of the isotope effect data for the double mutants with those of the general acid mutants allows a direct comparison of how the interactions of cationic and hydrogen-bonding residues with the phosphoryl group during catalysis affect the nature of the transition state for an enzymatic phosphoryl transfer reaction.

Materials and Methods

Synthesis of Compounds. Natural abundance *p*-nitrophenyl phosphate, [^{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 described previously.[21] [^{14}N]-*p*-Nitrophenol and [^{15}N,^{18}O]-*p*-nitrophenol were then mixed to reconstitute the natural abundance of ^{15}N, and then the mixture was phosphorylated to produce *p*-nitrophenyl phosphate using the same method as referred to above. This mixture was used for determination of $^{18}(V/K)_{bridge}$. The [^{14}N]-*p*-nitrophenyl phosphate and [^{15}N,nonbridge-$^{18}O_3$]-*p*-nitrophenyl phosphate were also mixed to reconstitute the natural abundance of ^{15}N. This mixture was used for determination of $^{18}(V/K)_{nonbridge}$. The isotopic abundance of the mixtures was determined by isotope ratio mass spectrometry.

Preparation of the Wild-Type *Yersinia* PTPase, D356A, D356N, R409K, and R409A and the Double Mutants R409A/D356A, R409K/D356N, R409K/D356A, and R409K/D356N. Site-directed mutagenesis of the *Yersinia* PTPase was performed according to the procedure of Kunkel,[22] using the Muta-Gene in vitro Mutagenesis kit

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from Bio-Rad. The oligonucleotide primers used for the desired substitutions were as follows: D356A, ATTGGCCCGCTCAGACCGC (primer 1); D356N, AATTGGCCCAATCAG ACCG (primer 2); R409A, GGTGTTGGCGCTACTGCGCAACT (primer 3); and R409K, GCGGGTGTTGGCAAAACTGCGCAA CTGAT (primer 4). The underlined base(s) indicate the change from the naturally occurring nucleotides. To prepare the double mutant R409A/D356A, both primer 1 and primer 3 were used in the mutagenesis experiments. Similarly, primers 2 and 3 were used for R409A/D356N, primers 1 and 4 for R409K/D356A, and primers 2 and 4 for R409K/D356N. All of the mutations were verified by DNA sequencing. The wild-type and the mutant recombinant *Yersinia* PTPases were expressed in *E. coli* and purified to homogeneity as described previously.[1,23]

Isotope Effect Determinations. Isotope effect determinations were made at 30 °C in 100 mM MES buffer, pH 6.0. The protocols for carrying out these experiments were the same as those previously described.[24] These involved running the enzymatic reactions to partial completion followed by separating the product and residual substrate, and subjecting these to isotopic analysis by isotope ratio mass spectrometry to determine the isotope effect. Isotopic analyses were performed using an ANCA-NT combustion system in tandem with a Europa 20−20 isotope ratio mass spectrometer. Reactions were begun with 100 μmol or more of the substrate and sufficient enzyme used so that the background hydrolysis rate was negligible compared to the enzymatic reaction. Parallel experiments without enzyme were used to establish background hydrolysis rates under the experimental conditions.

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). Equations 1 and 2 were used to calculate the observed isotope effect either from R_p and R_o or from R_s and R_o respectively at fraction of reaction f.[25] Thus each experiment yields two independent determinations of the isotope effect.

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

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

R_o was determined separately from unreacted substrate by isotope ratio mass spectroscopic analysis and, as a control, from *p*-nitrophenol isolated after complete hydrolysis of substrate using the isolation and purification procedures used in the isotope effect experiments. The agreement of these two numbers demonstrated that, within experimental error, no isotopic fractionation occurs as a result of the procedures used to isolate and purify the *p*-nitrophenol.

The ^{18}O isotope effects were measured by the remote-label method,[26] as previously described for the solution reactions of *p*NPP.[21] In these experiments the nitrogen atom in the substrate is used as a reporter for isotopic changes at the bridging oxygen atom or the nonbridging oxygen atoms. These experiments yield an observed isotope effect that is the product of the effect due to ^{15}N and to ^{18}O substitutions. The observed isotope effects from these experiments were then corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material as previously described.[27]

The notation used to express isotope effects is that of Northrop[28] where a leading superscript of the heavier isotope is used to indicate the isotope effect on the following kinetic quantity; for example, ^{15}k denotes k_{14}/k_{15}, the nitrogen-15 isotope effect on the rate constant k. Since all isotope effects in this study were measured by the competitive method they are isotope effects on V/K, and thus are designated as ^{15}(V/K), etc. Figure 2 shows a diagram of the substrate with the nomenclature used to describe the isotope effects at each position.

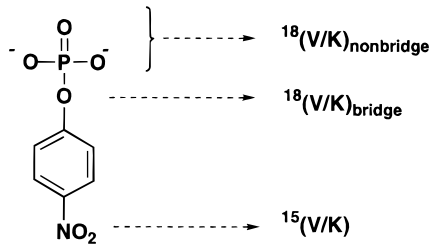

Figure 2. A diagram of the *p*-nitrophenyl phosphate substrate showing the positions at which isotope effects were labeled.

Kinetic Experiments with Mutant *Yersinia* PTPases. All enzyme assays were performed at 30 °C in buffers with a constant ionic strength of 0.15 M. Buffers used were as follow: pH 5.0−5.5, 100 mM acetate; pH 6.0, 50 mM succinate; pH 7.0, 50 mM 3,3-dimethylglutarate; and pH 8.0, 100 mM Tris. All of the buffer systems contained 1 mM EDTA and the ionic strength of the solutions was kept at 0.15 M using NaCl. Initial rate measurements for the enzyme-catalyzed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP, obtained from Fluka) were conducted as previously described.[1] The Michaelis−Menten kinetic parameters were determined from a direct fit of the velocity versus [S] data to the Michaelis−Menten equation using the nonlinear regression program KinetAsyst (IntelliKinetics, State College, PA). The range of *p*NPP concentration used was from 0.2 to 5 K_m. In all cases, the enzyme concentration was much lower than that of the substrate so that the steady-state assumption was fulfilled.

Results

Steady-state kinetic parameters were measured with the native *Yersinia* PTPase and its Asp356 and Arg409 mutants using *p*NPP as a substrate. At pH 5.0 and 30 °C the k_{cat} values for R409K and R409A were 0.026 and 0.072 s^{-1}, respectively, which were 46000- and 17000-fold lower than those for the native enzyme. The k_{cat}/K_m values for R409K and R409A were 0.0051 and 0.0011 s^{-1} mM^{-1}, respectively, which were 84000- and 390000-fold lower than those for the native enzyme.

The pH−rate profile for the wild-type Yersinia PTPase-catalyzed pNPP reaction is bell-shaped, indicating two enzymatic ionizations important for catalysis.[29] The group that must be unprotonated is likely the active site Cys residue and the group that must be protonated corresponds to the general acid. Previously it was shown that mutation to the general acid Asp356 eliminated the basic limb of the pH−rate profile observed in the wild-type enzyme-catalyzed reaction.[7,29] The k_{cat}/K_m values for D356N and D356A are identical (0.28 s^{-1} mM^{-1}) and are 4300-fold lower than the wild-type enzyme under optimal conditions (pH 5.0 and 30 °C). Interestingly, the R409K-catalyzed reaction shows no pH dependence from pH 4.8 to 8.0, while the R409A-catalyzed reaction requires a group with an apparent pK_a of 5.6 that must be protonated for optimal activity (Hoff et al., unpublished observation). These results suggest that Asp356 is not functional as a general acid in R409K. In contrast, the general acid may still be operative in R409A. Thus, mutations to the active site Arg409 can have an adverse effect on general acid function in addition to perturbing transition-state stabilization. The kinetic parameters for the double mutants, in which both Asp356 and Arg409 were altered,

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Table 1. Kinetic Parameters of the *Yersinia* PTPase and Its Asp356 and Arg409 Mutants

pH	*Yersinia* PTPase	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
5.0	WT	1200 ± 30	2.50 ± 0.20	480 ± 32
	D356A	0.17 ± 0.005	0.64 ± 0.04	0.26 ± 0.02
	D356N	0.85 ± 0.04	3.53 ± 0.35	0.24 ± 0.03
	R409A/D356A	0.014± 0.002	22 ± 3	0.00064 ± 0.00013
	R409A/D356N	0.016 ± 0.004	24 ± 4	0.00067± 0.00020
	R409K/D356A	0.017 ± 0.002	6.2 ± 1.4	0.0027 ± 0.0007
	R409K/D356N	0.014 ± 0.001	3.8 ± 0.7	0.0037 ± 0.0007
5.5	WT	670 ± 20	2.30 ± 0.12	290 ± 17
	R409A/D356A	0.017 ± 0.005	21 ± 2.6	0.00081 ± 0.00026
	R409A/D356N	0.011 ± 0.003	16 ± 1.9	0.00088 ± 0.00026
	R409K/D356A	0.014 ± 0.005	3.8 ± 0.4	0.0037 ± 0.0014
	R409K/D356N	0.011 ± 0.007	4.8 ± 0.9	0.0023 ± 0.0006
6.0	WT	340 ± 12	2.4 ± 0.14	140 ± 9
	D356A	0.16 ± 0.02	0.58 ± 0.12	0.28 ± 0.07
	D356N	0.91 ± 0.03	3.30 ± 0.29	0.28 ± 0.03
	R409A/D356A	0.016 ± 0.002	17 ± 0.8	0.00094 ± 0.00012
	R409A/D356N	0.014 ± 0.004	18 ± 1	0.00078 ± 0.00023
	R409K/D356A	0.016 ± 0.002	3.8 ± 0.19	0.0042 ± 0.0005
	R409K/D356N	0.013 ± 0.002	4.7 ± 0.5	0.0028 ± 0.0005
7.0	WT	35 ± 2	2.3 ± 0.11	157 ± 1.1
	D356A	0.14 ± 0.002	0.41 ± 0.12	0.34 ± 0.11
	D356N	0.89 ± 0.02	3.20 ± 0.24	0.28 ± 0.02
	R409A/D356A	0.020 ± 0.002	19 ± 0.6	0.0010 ± 0.0001
	R409A/D356N	0.015 ± 0.004	15 ± 1.1	0.0010 ± 0.0003
	R409K/D356A	0.015 ± 0.002	3.6 ± 0.12	0.0042 ± 0.0006
	R409K/D356N	0.012 ± 0.002	3.8 ± 0.2	0.0032 ± 0.0006
8.0	WT	3.4 ± 0.20	2.2 ± 0.30	1.5 ± 0.2
	R490A/D356A	0.013 ± 0.004	19 ± 2	0.00068 ± 0.00022
	R409A/D356N	0.012 ± 0.003	18 ± 1	0.00067 ± 0.00017
	R409K/D356A	0.015 ± 0.003	5.4 ± 0.3	0.0028 ± 0.0006
	R409K/D356N	0.013± 0.005	5.9 ± 0.4	0.0022 ± 0.0008

Table 2. Isotope Effects from Reactions with PNPP of Native *Yersinia* and Mutants

enzyme	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
native enzyme	0.9999(3)	1.0152(6)	0.9998(13)
D356N	1.0024(5)	1.0275(16)	1.0022(5)
D356A	1.0022(3)	1.0274(8)	1.0007(5)
R409K	1.0020(5)	1.0273(3)	1.0049(7)
R409A	1.0012(3)	1.0200(5)	0.9990(7)
R409K/D356N	1.0022(1)	1.0317(3)	1.0045(2)
R409K/D356A	1.0023(1)	1.0322(9)	1.0045(2)
R409A/D356N	1.0024(4)	1.0340(11)	1.0027(5)
R409A/D356A	1.0025(1)	1.0310(11)	1.0030(2)

were determined at pH 5.0, 5.5, 6.0, 7.0, and 8.0, and are summarized in Table 1. The values for the wild-type and D356N and D356A are included for comparison. As shown in Table 1, the reactions catalyzed by the double mutants are pH independent from pH 5.0 to 8.0, consistent with the absence of general acid catalysis. In addition, the kinetic parameters for the double mutants are similar to those of the R409K mutant, which supports the notion that general acid catalysis in R409K is abolished.

The isotope effect data for the reactions of the *Yersinia* PTPase mutants with *p*NPP are shown in Table 2 with their standard errors. The enzymatic substrate is the dianion of *p*NPP. Isotopic fractionation in the nonbridge oxygen atoms will affect the proportion of the isotopic isomers present as the dianion at this pH. The values for $^{18}(V/K)_{nonbridge}$ in Table 2 at pH 6.0 have been corrected for this effect as previously described.[21] The $^{18}(V/K)_{nonbridge}$ values shown are the isotope effects resulting from ^{18}O in all three nonbridge oxygen atoms. For purposes of comparison the isotope effects from previous studies of the reaction of the native enzyme, and the general acid mutants D356N and D356A, with *p*NPP are also shown.

Discussion

Background for Interpretation of the Isotope Effects. Kinetic isotope effects can characterize reactions in detail, in particular yielding information about the structure of the transition state. The transition state for hydrolysis of phosphate monoesters in solution can be described as very loose or "dissociative" in nature, characterized by extensive bond cleavage to the leaving group, minimal bond formation to the nucleophile, and in which the transferring phosphoryl group resembles metaphosphate ion.[10,11] Phosphodiesters and triesters exhibit successively tighter, more associative transition states characterized by less bond cleavage to the leaving group and greater bond formation to the nucleophile, where the transferring phosphoryl group resembles a pentacoordinate phosphorane.[11] Linear free energy relationships indicate that in diesters and triesters with good leaving groups, these reactions are concerted with no phosphorane intermediate, but that the transition states become tighter (more associative) than in the dissociative transition state of the monoester reaction.[30,31]

Isotope effects have been measured for the phosphoryl transfer reactions of a number of phosphate esters in solution.[21,27,32−35] The cumulative data indicate that isotope effects can distinguish between these types of transition states and therefore should be able to detect a significant enzyme-induced change to the structure of the transition state. Calculations predict inverse nonbridge ^{18}O isotope effects for dissociative transition states, and normal values for associative transition states.[35] The experimental nonbridge ^{18}O isotope effects for uncatalyzed reactions of the *p*NPP monoester are small and inverse (0.9994−0.9997), while the nonbridge ^{18}O isotope effects for diesters and triesters which have been measured are (with a single exception, which may be anomalous) normal (1.0040 to 1.0250). The isotope effects in the leaving group also distinguish between the loose transition states of monoesters and the tighter ones of diesters and triesters, and echo the conclusions from linear free energy relationships that bond cleavage to the leaving group is significantly less in the latter reactions. The isotope effects $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ measure charge delocalization in the leaving group and P−O bond cleavage, respectively. The extensive bond cleavage in reactions of the *p*NPP dianion result in bridge-^{18}O isotope effects of from 1.0202 to 1.030; the resulting development of nearly a full negative charge on the leaving group results in ^{15}N isotope effects of from 1.0028 to 1.0039. The tighter transition states of diesters and triesters are characterized by less bond cleavage to the leaving group, and exhibit reduced values for bridge-^{18}O isotope effects, in the range of 1.0039 to 1.0060, as well as smaller ^{15}N isotope effects, which range from 1.0007 to 1.0016.

When protonation of the leaving group occurs in the transition state the normal $^{18}(V/K)_{bridge}$ isotope effect arising from P−O bond cleavage is reduced by the inverse isotope effect arising from protonation. This is clearly observed by comparing the reactions of native PTPases where protonation of the leaving group is accomplished by a conserved Asp general acid, with

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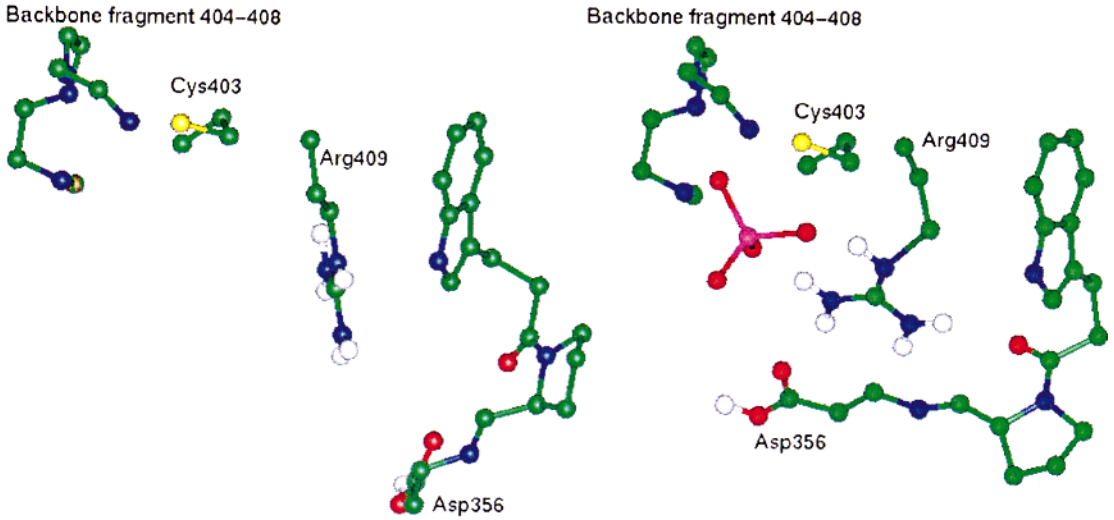

Figure 3. The orientations of groups at the active site of the *Yersinia* PTPase in the unbound state (left) and when sulfate is bound at the active site (right). Other oxyanions such as tungstate or phosphate result in similar conformational changes. Upon binding of oxyanions, Arg409 rotates to form two hydrogen bonds to the anionic group, resulting in the formation of a new hydrogen bond with the carbonyl oxygen atom of Trp354. Associated movement of the WpD loop brings Asp356 into position to function as a general acid during catalysis. The structures shown are from published X-ray structures[6,53] with computer-generated hydrogen atoms added to the Arg and Asp residues.

data from general acid mutants. The maximum $^{18}(V/K)_{bridge}$ effect seen in reactions of *p*NPP in solution in which the leaving group is not protonated is around 1.03, and the same value is observed in PTPases when general acid catalysis has been eliminated by mutation.[24,36,37] The equilibrium ^{18}O isotope effect for protonation of *p*-nitrophenol is 0.985.[38] Thus when P−O bond cleavage and proton transfer are both extensive and synchronous, the observed $^{18}(V/K)_{bridge}$ isotope effect should be close to the product of these values, or about 1.015. This is close to the value measured in reactions of *p*NPP with PTPases where protonation of the leaving group occurs in the transition state.[24,36,37]

The kinetic isotope effects for the reaction of the native *Yersinia* PTPase with *p*NPP were measured in a previous study, and are most consistent with a dissociative transition state with both P−O bond cleavage and protonation of the leaving group well-advanced.[24] Mutation of the general acid Asp356 results in an enzyme-catalyzed reaction in which the leaving group departs as the negatively charged nitrophenolate. This reaction is also characterized by a very late transition state with respect to bond cleavage. The general acid mutant reactions also exhibits $^{18}(V/K)_{nonbridge}$ isotope effects indicative of more nucleophilic participation in the transition state than seen in the reaction of *p*NPP dianion in solution, but less than that seen in diesters or triesters.[24]

With this framework of background data, the isotope effects for the *Yersinia* mutants in the present study can be evaluated for the effect of the mutations on the transition state of the phosphoryl transfer reaction.

Does the Presence of the Arg Residue Favor an Associative Transition State? (a) Results from Arg409 Single Mutants. At pH 6 and 30 °C, the k_{cat} values for R409K and R409A are 0.017 and 0.040 s^{-1}, respectively, and the K_m values for R409K and R409A are 4.8 and 68 mM, respectively. Under the same conditions, the k_{cat} and K_m for the wild-type are 340 s^{-1} and 2.4 mM. These catalytic rates are approximately 10^4 less than the native enzyme but are still nearly 10^5-fold faster than the uncatalyzed rate for aqueous hydrolysis of the dianion of *p*NPP. There is little pH dependence for the R409K mutant from pH 4.8 to 8.0. The isotope effects in the leaving group, $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$, both are changed from their values in the reaction of the native enzyme and resemble the values in the reaction of the general acid mutant D356N, indicating that in the catalytic reaction of R409K the leaving group departs with substantial negative charge. Collectively, results from the pH dependence experiments and the isotope effects on the reaction with R409K indicate that the Arg-to-Lys mutation prevents the functioning of the general acid.

X-ray structures of the enzyme with and without bound substrate analogues provide an explanation for why mutation of the Arg409 residue should affect the function of the general acid (Figure 3). The general acid resides on a flexible loop that in the absence of bound substrate places the Asp356 residue approximately 8 Å from the active site. X-ray structures show that when an oxyanion such as sulfate is bound, the Arg409 residue moves to form bidentate hydrogen bonds with two oxygen atoms of the anion.[39] Structural changes in the active site result in the formation of a new hydrogen bond between Arg409 and the carbonyl oxygen atom of Trp354 (Figure 3). As a result, the indole ring of Trp354 moves into a hydrophobic crevice, making van der Waals contacts with the aliphatic portion of Arg409.[39] These interactions are associated with movement of the loop to attain the closed position when the oxyanion is bound, which brings the general acid into position to assist catalysis. Indeed, when Trp354 is replaced by either a Phe or an Ala, both the function of the general acid and the ability of the Arg409 to bind oxyanions are affected,[40] indicating a functional coupling between Asp356 and Arg409 through residue Trp354. The results indicate that in R409K the lysine residue does not interact with the substrate and Trp354 in a productive fashion, thus preventing the proper alignment between the leaving group of the substrate and the general acid Asp356.

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For the reaction of R409A at pH 6.0, each of the isotope effects $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ are intermediate between those for D356N and those for the native enzyme where protonation of the leaving group in the transition state is intact. This is consistent with a reaction in which partial proton transfer occurs in the transition state, but where a partial negative charge develops on the leaving group as a result of the extent of protonation lagging behind P−O bond cleavage. Further evidence for this is the observation that the R409A-catalyzed reaction displays a pH dependency similar to the basic limb of the native enzyme reaction (Hoff et al., *Biochemistry*, submitted for publication).

If Arg409 imposes associative character to the reaction catalyzed by the wild-type enzyme, then elimination of the guanidinium group should lead to a significantly decreased (or more inverse) $^{18}(V/K)_{nonbridge}$ isotope effect. This is not observed in the reactions with either R409K or R409A. In the R409A reaction, in which general acid catalysis is still partially operative but the interactions between the guanidinium group and the phosphoryl oxygens are completely eliminated, the $^{18}(V/K)_{nonbridge}$ isotope effect is unchanged from that observed with reaction of the native enzyme (Table 2). This suggests that the function of Arg409 is to stabilize the transition state, not to dictate the nature of it.

However the data from the Arg409 mutants (especially R409K) cannot be clearly interpreted solely in terms of the effects of interactions between this residue and the phosphoryl group on the structure of the transition state, since mutation of this residue adversely affects general acid catalysis. Loss of general acid catalysis itself causes alterations to the transition state, requiring the leaving group to depart as an anion which results in a small increase in nucleophilic participation. Therefore double mutants were prepared in which Arg409 was changed to either Lys or Ala while the general acid was mutated either to Asn or Ala. In these double mutants the leaving group necessarily departs as the anion, and the effect of mutations to the Arg residue can be evaluated free of the workings of the general acid.

(b) Results from Arg409 and Asp356 Double Mutants. As expected, the reactions catalyzed by the Arg409 and Asp356 double mutants exhibited minimal pH dependency between pH 5.0 and pH 8.0 (Table 1). Furthermore, comparison of the kinetic parameters of the general acid single mutants with those of the double mutants allowed a direct assessment of the role of Arg409 in PTPase catalysis. The k_{cat}/K_m values of D356A and D356N are 300−360-fold higher than those of R409A/D356A and R409A/D356N, suggesting that the side chain of Arg409 contributes 3.4−3.5 kcal/mol to transition-state stabilization. In addition, comparison of the k_{cat}/K_m values of R409K/D356A and R409K/D356N with those of R409A/D356A and R409A/D356N indicates that the Lys residue is only 4 times better than an Ala in substituting for Arg409 in PTPase catalysis.

The loss of general acid catalysis has the effect of creating a poorer leaving group, by raising the pK_a of this group in the transition state. The Hammond postulate predicts that such a change should make the transition state tighter than in the reaction of the native enzyme, with increased nucleophilic participation. Evidence that this should indeed be the case comes from the isotope effects for reactions of general acid mutants. In all of the PTPases examined in previous studies the loss of general acid catalysis resulted in small normal nonbridge-^{18}O isotope effects, indicative of an increase in nucleophilic participation in the transition state. While these isotope effects were smaller in magnitude than the normal isotope effect at this position in the associative reactions of phosphate diesters and triesters, the change is significant and consistent among all PTPases.[24,36,37] Linear free energy data from uncatalyzed phosphoryl transfer reactions in solution also indicate that increased basicity of the leaving group results in increased nucleophilic participation in the transition state.[41,42] An enforced increase in nucleophilic participation in the transition state should enhance the importance of the cationic Arg residue in catalysis if indeed this residue serves to assist in stabilization of increased negative charge in an associative transition state. Thus, while not testing the effect on the transition state of the "natural" enzymatic reaction, the double mutant experiments nonetheless constitute a valid test of the proposition that the positive charge and hydrogen-bonding interactions of Arg409 alter the nature of the transition state in addition to providing for its stabilization. Without general acid catalysis, the pK_a of the leaving group is probably higher in the transition state of the reaction with pNPP than in reactions of natural phosphotyrosyl substrates with the native enzyme, where protonation concerted with P−O bond cleavage keeps the tyrosyl oxygen atom neutral in the transition state.

A comparison of the isotope effect data for the double mutant reactions with those for the general acid mutants reveals the effect of the Arg409 residue on the structure of the transition state (Table 2).

The ^{15}N isotope Effects. The secondary ^{15}N isotope effect measures charge development on the *p*-nitrophenol leaving group. Its typical value is 2- to 3-fold higher in dissociative mechanisms than in the associative reactions of triesters and diesters. The values for $^{15}(V/K)$ for all of the double mutants are identical within experimental error with the data from the general acid single mutants. This indicates that no change in the amount of negative charge developed on the leaving group results from mutating Arg409 to either Lys or Ala.

The Bridge-^{18}O Isotope Effects. This isotope effect is sensitive to the extent of P−O bond cleavage. The $^{18}(V/K)_{bridge}$ isotope effects are slightly larger in the reactions of the double mutants than in those of the general acid mutants. These changes are in the direction expected if the cationic Arg residue favors a more associative transition state, which becomes more dissociative when this residue is mutated. However the observed increases are very small; by comparison, this isotope effect typically varies by from 4- to 10-fold between reactions with loose transition states compared with reactions of diesters and triesters which have tighter transition states. The large magnitudes of this isotope effect in the reactions of the general acid mutants indicate that bond cleavage to the leaving group is extensive. At most, this transition-state parameter may be slightly greater in the double mutants. The lack of a change in the $^{15}(V/K)$ isotope effect also indicates that changes in bond cleavage to the leaving group resulting from mutations to Arg409 must be small. Significantly more advanced P−O bond cleavage would necessarily result in greater negative charge on the leaving group in reactions of these double mutants, which would manifest itself by an increase in the magnitude of $^{15}(V/K)$.

The Nonbridge-^{18}O Isotope Effects. Comparisons of the $^{18}(V/K)_{nonbridge}$ isotope effects of the double mutants with those of the general acid mutants also indicate that little change to the transition-state structure occurs as a result of mutations to Arg409. The magnitude of the $^{18}(V/K)_{nonbridge}$ isotope effect

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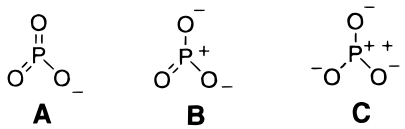

Figure 4. Resonance structures of the metaphosphate ion.

is slightly higher in each of the double mutant reactions than in the reactions of the general acid mutants. These small increases are in the opposite direction than expected if the Arg409 residue imparts associative character and the reaction becomes more dissociative in its absence; in this case this isotope effect should move in the inverse direction, not become more normal as is observed. The magnitude of the change again is very small relative to the range of values exhibited by this isotope effect in the continuum of tight versus loose transition states of phosphate esters.

In summary, the lack of significant changes in the isotope effects indicates that at most small perturbations to the transition-state structure are caused by the interactions of the substrate with Arg409 during catalysis. These results indicate that the function of this residue is stabilization of the transition state, and that it does not significantly alter it.

These results echo data from reactions in solution where the presence of metal ions has been shown by linear free energy relationships not to increase the associative character of the transition states of GTP hydrolysis[43] or of the reactions of phosphorylated pyridines.[44] In addition, linear free energy data show that large changes in reactivity result in only small changes in the transition state for phosphoryl transfer, suggesting that this transition state is resistant to change.[41,45,46]

How, Then, Can the Presence of Positive Charge at the Active Site of a Phosphoryl Transfer Enzyme Be Reconciled with a Dissociative Transition State? The seeming inconsistency arises from the perception that the cationic residue or metal ions are in proximity to a transferring group that loses negative charge in the transition state. Some of this perception arises from the classical representation of metaphosphate. In a dissociative transition state the transferring phosphoryl group will resemble this species, which is usually drawn as structure **A** in Figure 4. Computational results suggest that a more accurate view is that of a resonance hybrid with contributions from all three structures shown, with structures **B** and **C** the major contributors.[47,48] If this description is correct, then although the net negative charge of the phosphoryl group decreases, the charge on the nonbridging oxygen atoms will not undergo a significant change between the ground state and the transition state for phosphoryl transfer. Other means by which dissociative phosphoryl transfer might be stabilized in an enzymatic active site have been discussed.[43,49]

Conformational changes which are undergone by both the substrate and the enzyme during catalysis can allow cationic and hydrogen-bonding residues to interact more strongly with the transition state than with the ground state. The conformational changes of the *Yersinia* enzyme that result from the binding of oxyanions have already been mentioned. The phosphoryl group undergoes inversion during the catalytic reaction, changing from tetrahedral to planar in the transition state, with an accompanying increase in the O−P−O bond angle (Figure 1). Structural and computational studies have found that significant transition-state stabilization of the dissociative transition state by the PTPases can occur via hydrogen-bonding interactions that are enforced as a result of the spatial expansion of the three nonbridging oxygen atoms in going from the tetrahedral reactant state to a trigonal bipyramidal geometry at the transition state.[5,6]

Are Interatomic Distances in Complexes between Enzymes and Transition-State Analogues Reliable Indicators of Corresponding Distances during Catalysis? Isotope effect measurements are a direct probe of the transition state during a catalytic reaction. Do the conclusions from isotope effect studies agree with inferences from X-ray structures of PTPases complexed with transition-state analogues? Such structures can give valuable insights concerning the roles of catalytic groups in the transition state but are not probes of the reaction itself. Interatomic distances in such complexes are sometimes used to infer bond orders to the leaving group and/or the nucleophile in the transition state of the phosphoryl transfer reaction. However, there are limits to the certainty with which interatomic distances in such complexes can be translated into bond orders in the transition state. The structure of the *Yersinia* PTPase complexed with the transition state analogue vanadate shows a trigonal bipyramid with an axial V−S distance of 2.52 Å.[50] Using Pauling's rule, a bond order of 0.21 was calculated for the P−S bond to the nucleophilic Cys in the transition state using 2.12 as a single P−S bond length and assuming that the S−V distance accurately represents the S−P distance in the transition state for catalysis.[16] Small uncertainties in the interatomic distance translate into large uncertainties in the bond order, however, due to the logarithmic nature of the relationship between these quantities. Assuming an uncertainty of 10%, or ±0.22 Å, in this distance in the 2.2 Å X-ray structure gives a bond order range of 0.09−0.50 calculated from Pauling's rule. This uncertainty is too large to allow one to distinguish between a dissociative versus an associative mechanism. It must also be remembered that these enzymes undergo conformational changes during catalysis, and there is no assurance that distances in a ground-state complex with a transition-state analogue will match the corresponding distances during catalysis when the atoms involved are in motion.

The low molecular weight PTPases share the relative positions of the essential cysteine and arginine residues in the active site sequences and use an identical catalytic sequence as other PTPases.[51] In the crystal structure of a low molecular weight PTPase complexed with vanadate a S−V distance of 2.16 Å was found,[52] much smaller than the corresponding distance in the complex with the *Yersinia* enzyme. One might infer from this information that a much more associative transition state for catalysis, more resembling that typical of a phosphotriester, is operative in the low molecular weight PTPases than in the *Yersinia* enzyme. This would result in large differences between the isotope effects for the two enzymatic reactions, on a scale similar to the differences observed between reactions of monoesters and those of diesters or triesters. The isotope effects

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of one member of this family, yeast Stp-1, have been measured.[36] Only small differences were found in the transition state for the Stp-1-catalyzed reaction compared to that of the *Yersinia* PTPase. The two reactions show the same degree of bond cleavage to the leaving group, with the Stp-1-catalyzed reaction exhibiting slightly more nucleophilic participation in the transition state. These results suggest that factors unrelated to those at work during catalysis can affect the interatomic distances in complexes of enzymes with transition-state analogues.

Conclusions

The isotope effect results indicate that in the absence of general acid catalysis, the transition state for the phosphoryl transfer reaction catalyzed by the *Yersinia* enzyme is not significantly altered by interactions of the phosphoryl group with Arg409. Although the loss of transition-state stabilization provided by this residue results in a large decrease in rate, the results provide no support for the proposal that the presence of positive charge or hydrogen bonds necessarily results in a change to an associative mechanism. The interplay of the interactions of substrate with Arg-409 and the functioning of general acid catalysis made it impossible to examine the effect of mutations to this residue in the "natural" acid-catalyzed reaction. However, the removal of protonation of the leaving group should result in a more associative mechanism and thus enhance the importance of interactions with the conserved arginine if they alter, instead of simply stabilize, the transition state. The results indicate that no substantial alteration to the transition state takes place as a result of these interactions. This conclusion is consistent with computational studies that show that the active sites of PTPases stabilize a dissociative transition state. Comparison of isotope effect data from reactions of PTPases with data from X-ray structures of the enzymes with bound transition-state analogues suggests that interatomic distances in such complexes may not reliably report on corresponding distances in the transition state for catalysis.

Acknowledgment. Financial support of this work came from NIH grant GM47297 to A.C.H., from the U.S. Army Advanced Civil Schooling Program for support of R.H.H., and from NIH grant CA69202 to Z.Y.Z. Z.Y.Z. is a Sinsheimer Scholar and an Irma T. Hirschl Career Scientist. The authors thank Dr. Dan Herschlag for helpful comments.

JA992361O