Reactivity of Alcohols Toward the Phosphoenzyme Intermediate in the Protein-Tyrosine Phosphatase-Catalyzed Reaction: Probing the Transition State of the Dephosphorylation Step†

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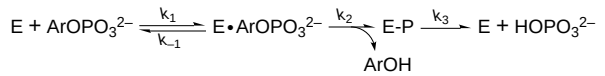
ABSTRACT: In solution phosphate monoesters hydrolyze via a highly dissociative mechanism involving a "loose" or "exploded" metaphosphate-like transition state where bond formation to the incoming nucleophile is minimal and bond breaking between phosphorus and the leaving group is substantial. To better understand how protein-tyrosine phosphatase (PTPase) effects catalysis, it is important to determine the nature of the enzymic transition state. PTPases catalyze the hydrolysis of phosphate monoesters by a two-step mechanism that proceeds through a phosphoenzyme intermediate (E-P). Extensive heavy atom kinetic isotope effect and leaving group dependency studies have provided insights into the nature of the transition state for the first step (E-P formation) of the PTPase reaction. In this paper we have probed the transition state for the low M_r PTPase-catalyzed dephosphorylation step by studying the effect of changing the alcohol basicity on its reactivity toward E-P. The Brønsted $\beta_{nu:}$ value for the reactions of alcohols and E-P is determined to be 0.14, which indicates that the enzymic transition state is highly dissociative and similar to that in uncatalyzed solution reactions. We show that the conserved hydroxyl group in the PTPase signature motif is primarily involved in the E-P dephosphorylation step. We further demonstrate that elimination of the hydroxyl group renders the transition state for E-P dephosphorylation *less* dissociative, suggesting that the main function of the hydroxyl group in the PTPase active site is to promote the E-P going through a dissociative pathway and to stabilize the dissociative transition state.

There is intense interest in protein-tyrosine phosphatases (PTPases)[1] due to their involvement in a multitude of physiological processes including cell growth and proliferation, cell cycle control, cytokine activation, and cytoskeletal integrity (Hunter, 1995; Barford et al., 1995). The PTPases constitute a family of enzymes (now >50 members) that rival the protein-tyrosine kinases in terms of structural diversity and complexity. PTPases catalyze the hydrolysis of phosphate monoesters (including phosphotyrosine) via a two-step, double-displacement mechanism (Scheme 1; Zhang & Dixon, 1994). In the first chemical step (k_2, phosphorylation) the active site Cys residue attacks the phosphorus atom in a substrate ($ArOPO_3^{2-}$) to expel the leaving phenol (ArOH), resulting in the formation of a covalent thiophosphate enzyme intermediate (E-P). In the second chemical step (k_3, dephosphorylation), a water molecule attacks the phosphorus atom of E-P to yield inorganic phosphate ($HOPO_3^{2-}$). It appears that under most conditions the rate-limiting step for the PTPases-catalyzed reaction corresponds to the decomposition of E-P (k_3) (Zhang & Van Etten, 1991; Zhang, 1995; Zhang et al., 1995a−c).

Scheme 1



Enzymes effect catalysis by lowering the energy of a transition state relative to a ground state. A better understanding of the nature of the transition state for the PTPase-catalyzed reaction should provide important insights into how PTPase carries out its function. Two mechanistic extremes can be considered. In an associative mechanism, the bond from the entering nucleophile is formed prior to bond fission to the leaving group, resulting in the formation of a pentavalent phosphorane intermediate. In a dissociative mechanism, the bond to the leaving group is broken before the bond to the nucleophile is made, resulting in the formation of a metaphosphate intermediate. Leaving group dependence (Zhang, 1995; Zhang et al., 1995c; Wu & Zhang, 1996) and heavy atom kinetic isotope effect (Hengge et al., 1995, 1996) studies have revealed a great deal of information about the transition state for the PTPase-catalyzed phosphorylation step (k_2). The transition state for the E-P formation step has been shown to be highly dissociative, and, as a result, there is negative charge developing on the departing phenolic oxygen atom. An Asp residue has been implicated to play the role of a general acid in the PTPase-catalyzed reaction (Zhang et al., 1994a; Zhang, Z., et al., 1994; Taddei et al., 1994; Denu et al., 1995). We have suggested that PTPases promote and stabilize the dissociative transition state by an advanced proton transfer from the active site Asp residue to the leaving group (Zhang et al., 1995b; Hengge

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[1] Abbreviations: PTPase, protein-tyrosine phosphatase; Stp1, small tyrosine phosphatase; *p*NPP, *p*-nitrophenyl phosphate; E-P, covalent phosphoenzyme intermediate; VHR, VH1-related.

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et al., 1995; Wu & Zhang, 1996). Little is known about the transition state for the dephosphorylation step (k_3). Thus it is not clear whether a similar strategy is utilized for effective transition state stabilization in the two chemical steps.

In this paper, we have investigated the PTPase-catalyzed E-P dephosphorylation (k_3). The system being studied is the low M_r PTPase from the fission yeast (*Schizosaccharomyces pombe*), Stp1, which is a high-copy suppressor of *cdc25*−*22*, implicating that Stp1 may play a role in cell cycle regulation (Mondesert et al., 1994). Previously, we have shown that Stp1 is a good model for detailed kinetic and mechanistic characterization of the low M_r PTPase-catalyzed reaction (Zhang et al., 1995a; Wu & Zhang, 1996). Since low M_r PTPases also catalyze phosphotransfer reactions, we have probed the transition state for the dephosphorylation (k_3) step by studying the effect of changing the basicity of the alcohol nucleophiles on the reactivity of the alcohols toward the phosphoenzyme intermediate. Our results suggest that, like the phosphorylation step, the transition state for the breakdown of E-P is also highly dissociative. We also show that the side chain of Ser18 in the PTPase signature motif of Stp1 plays an important role in facilitating the breakdown of E-P. Strikingly, elimination of the conserved hydroxyl group substantially alters the nature of the transition state for the dephosphorylation step.

MATERIALS AND METHODS

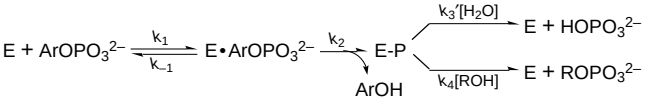
Materials. *p*-Nitrophenyl phosphate (*p*NPP) was from Fluka. Ethyl alcohol was from Pharmco. Methyl alcohol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, *tert*-butanol, 2-methoxyethanol, 2-chloroethanol, allyl alcohol, 2,2,2-trifluoroethanol, and propargyl alcohol were from Aldrich. 2-Cyanoethanol was from Sigma. Mutagenesis kits were from Bio-Rad. DNA sequencing kit was from USB.

Site-Directed Mutagenesis and Construction of Plasmids. Mutagenesis of residue Ser18 of the low molecular weight protein phosphatase Stp1 (small tyrosine phosphatase) was performed as described previously (Wu & Zhang, 1996). The plasmid pUC118-Stp1 (Zhang et al., 1995a) was used to make single-stranded DNA for site-directed mutagenesis. The oligonucleotide primer used for the Ser18 to Ala mutation was 5′-CATTTGCAGAGCTCCAATGGCTG-3′. The underlined bases indicate the change from the naturally occurring nucleotides. The mutation was confirmed by DNA sequencing. The DNA fragment of Stp1/S18A was subcloned into pT7-7 vector as was done for Stp1 wild-type gene (Zhang et al., 1995a).

Expression and Purification of Recombinant the Wild-Type Stp1 and the Mutant S18A. The wild-type Stp1 and mutant S18A were expressed in *Escherichia coli* strain BL21 (DE3) and purified to homogeneity as described (Zhang et al., 1995a), except that *E. coli* expressing S18A was grown overnight at room temperature after induction with 0.4 mM isopropyl β-D-thiogalactoside (IPTG), since the majority of the protein was insoluble when expressed at 37 °C.

Steady-State Kinetics. The assay for the hydrolysis of *p*NPP by the wild-type Stp1 and the mutant S18A was carried out as previously described (Zhang et al., 1995a). The assay buffer was 50 mM succinate and 1 mM EDTA at pH 6.0 with ionic strength of 0.15 or 1.0 M. The reaction was initiated by addition of enzyme and quenched after 1−2 min by addition of 1 mL of 1 M 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 $M^{-1}cm^{-1}$. Michaelis−Menten kinetic parameters were determined from a direct fit of the velocity vs [S] using the nonlinear regression program Kinetasyst (IntelliKinetics, State College, PA).

Scheme 2



Pre-Steady-State Kinetics. Rapid kinetic measurements of hydrolysis of *p*NPP by the wild-type Stp1 and the mutant S18A at 30 °C were performed as described (Zhang et al., 1995a). The final concentration of *p*NPP was 10 mM. The concentration of Stp1 and S18A was 56.7 and 59.5 μM, respectively. The buffer used was 50 mM succinate and 1 mM EDTA, pH 6.0, with ionic strength maintained at 1.0 M by adding NaCl. High ionic strength was necessary to keep the S18A from precipitating out of the solution during the stopped-flow experiments at high enzyme concentrations.

Determination of the Second-Order Rate Constants for the Phosphoryl Transfer Reaction from the Phosphoenzyme Intermediate to Various Alcohols. The rate-limiting step for the Stp1-catalyzed hydrolysis of *p*NPP is the breakdown of the phosphoenzyme intermediate (Zhang et al., 1995a). Thus, the observed k_{cat} values for *p*NPP processing in the presence of alcohols as determined by following the production of *p*-nitrophenol are the sum of the individual rate constants for the E-P hydrolysis (phosphoryl transfer from E-P to H_2O) and alcoholysis (phosphoryl transfer from E-P to ROH). A plot of k_{cat} against alcohol concentration is linear with an intercept at the rate of hydrolysis. The slope of this line is the second order rate constant for the reaction of E-P with alcohol.

RESULTS AND DISCUSSION

Stp1 Catalyzes Phosphoryl Transfer Reaction from E-P to Alcohols. The low M_r PTPase-catalyzed hydrolysis of aryl phosphates has been shown to yield, in addition to the hydrolysis product (inorganic phosphate), alkyl phosphates in the presence of alcohols (Zhang & Van Etten, 1991; Wu & Zhang, 1996). We have also observed the production of ethyl phosphate in the Stp1-catalyzed hydrolysis of *p*NPP in the presence of ethanol by ^{31}P NMR (data not shown). This presumably results from partitioning of the phosphoenzyme intermediate (E-P) between its reaction with water to give the hydrolysis product and its reaction with alcohols to produce the alkyl phosphates (Scheme 2). The fact that the ratio of products (inorganic phosphate to alkyl phosphate) is independent of the leaving group identity (Zhang & Van Etten, 1991) indicates that reactions with water and alcohols emanate from E-P. Scheme 2 illustrates the partitioning of E-P in the presence of alcohol ROH, in which k_3 (= $k_3'[H_2O]$) is the rate of hydrolysis while k_4[ROH] is the rate of E-P reacting with ROH to form the phosphorylated alcohol ($ROPO_3^{2-}$). The observation that the breakdown of E-P and hence the overall reaction rate is increased in the presence of an alternative nucleophile is consistent with the rate-limiting step being the hydrolysis of the intermediate. Thus, information about the transition state of E-P dephosphorylation can be obtained by studying the selective influence

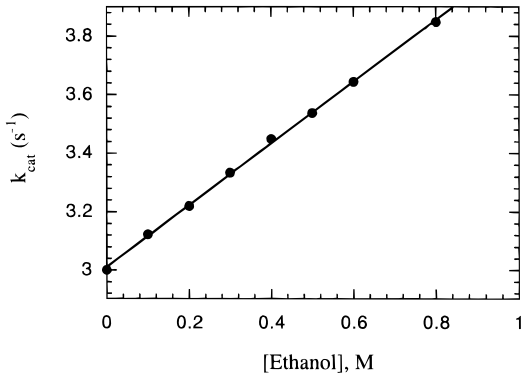


FIGURE 1: Effect of ethanol concentration on Stp1 k_{cat} at pH 6.0 and 30 °C.

Table 1: Second Order Rate Constants for Reaction of Alcohols with E-P at 30 °C and pH 6.0

alcohols	pK_a	k_4 (M^{-1} s^{-1})
methyl alcohol	15.5	2.67 ± 0.03
ethyl alcohol	15.9	1.05 ± 0.03
n-propyl alcohol	16.1	2.19 ± 0.09
iso-propyl alcohol	17.1	0.80 ± 0.05
n-butyl alcohol	16.1	4.79 ± 0.11
sec-butyl alcohol	17.6	1.00 ± 0.05
tert-butyl alcohol	19.2	0.65 ± 0.03

[a] The pK_a values were obtained as described in the following references (Ballinger & Long, 1960; Murto, 1964; Takahashi et al., 1971).

of a nucleophilic acceptor on the partitioning of E-P in the Stp1-catalyzed *p*NPP hydrolysis. The $\beta_{nu:}$ parameter, obtained from structure−reactivity correlation of reactivity as a function of the pK_a of the attacking nucleophile, may be viewed as an empirical index of the fraction of charge transferred to the nucleophile and, correspondingly, may reflect the degree of bond formation between the nucleophile and the phosphorus in the transition state (Jencks & Gilchrist, 1968).

In experiments with Stp1 and *p*NPP conducted in the presence of alcohol, the overall rate of substrate turnover k_{cat} as measured by the production of *p*-nitrophenol is given by eq 1. Since the rate-limiting step is predominantly

$$k_{cat} = \frac{k_2(k_3 + k_4[\mathrm{ROH}])}{k_2 + k_3 + k_4[\mathrm{ROH}]} \quad (1)$$

$$k_{cat} = k_3 + k_4[\mathrm{ROH}] \quad (2)$$

determined by the decomposition of E-P (Zhang & Van Etten, 1991; Zhang et al., 1995a), eq 1 can be reduced to eq 2, from which the second-order rate constant k_4 can be determined.

Figure 1 shows the linear increase in k_{cat} for reaction of Stp1 with *p*NPP in the presence of increasing concentrations of ethanol. The rate constant for hydrolysis of E-P (k_3) is obtained as the intercept, and the second-order rate constant for the phosphoryl transfer from E-P to ethanol (k_4) is obtained as the slope. Similar plots are obtained for all of the alcohols examined. Table 1 summarizes the second-order rate constant (k_4) for the alcoholysis of E-P. There are three conclusions that can be drawn from the table. First, the reactivity of the alcohols is decreased by steric hindrance, as shown by the lower k_4 values of secondary and tertiary alcohols than those of primary alcohols, despite of the fact

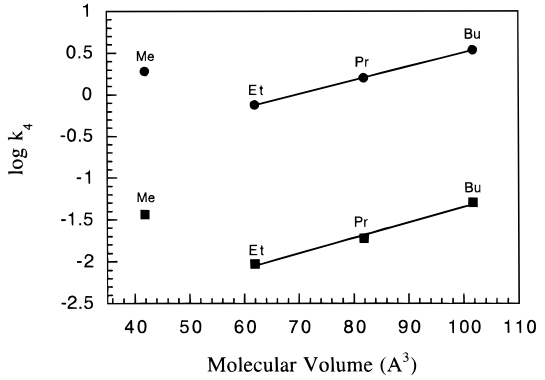


FIGURE 2: Relationship between log k_4 and molecular volume ($Å^3$) for methyl (Me), ethyl (Et), propyl (Pr), and butyl (Bu) alcohols, (●) wild type Stp1 and (■) Stp1/S18A. The pK_a values for ethyl, propyl, and butyl alcohols are similar, so that only changes in the molecular volume affect the rate constant. The molecular volumes of the alcohols were calculated by first building and then energy-minimizing the molecules, using the molecular modeling program INSIGHT II (Biosym Technologies).

that secondary and tertiary alcohols have higher pK_a's than primary alcohols. This is consistent with the nature of the reaction being nucleophilic and the observed sensitivity to steric effects in phosphate ester substitution reactions (Kirby & Jencks, 1965). Second, the reactivity of primary alcohols, such as ethanol, propanol, and butanol, increases as the length of the aliphatic chain increases. This is also evident from a plot of log k_4 versus solvent-excluded molecular volume shown in Figure 2. Since the pK_a value and thus the chemical reactivity of these alcohols change little as the chain length becomes longer, this result requires a specific interaction between the alcohol and the enzyme active site which may be hydrophobic in nature. This is consistent with the observations that low M_r PTPases prefer bulky substrates (Zhang et al., 1995a) and that residues lining the catalytic site are largely hydrophobic (Zhang, M., et al., 1994). It is possible that the enhanced reactivity of the longer alcohols results from the increased binding energy between the alcohol and the enzyme. Interestingly, methanol is an exception to this in that it is more reactive than ethanol. It is important to recognize that, for reactions of phosphate with ethanol, propanol, and butanol, the steric factor or the crowdedness on the α carbon is similar for all of them, whereas for the reaction of phosphate with methanol the steric factor is much more favorable. Thus, the rate-diminishing effect of decreasing chain length on going from ethanol to methonal is more than offset by the decreased steric hindrance to nucleophilic attack for methanol relative to ethanol. Thirdly, it appears that alcohols are better phosphoacceptors than water in the Stp1-catalyzed reaction. This point is discussed further below.

Alcohols Are Better Nucleophiles in Stp1-Catalyzed Phosphoryl Transfer Reaction. The second-order rate constant for the hydrolysis of E-P (k_3') in the Stp1-catalyzed reaction is 0.054 M^{-1} s^{-1}, which is calculated by dividing k_3 (3.0 s^{-1}) by the bulk concentration of water (55.5 M). If one compares the second-order rate constant for alcoholysis of E-P (k_4) with that of hydrolysis (k_3'), one may conclude that alcohols are much better nucleophiles than water in the Stp1-catalyzed reaction. This is also seen from the fact that the effect of alcohols on the dephosphorylation reaction can be measured at low concentrations (<1 M) in aqueous solution. The pK_a value for water is 15.7 and those for primary alcohols are around 16. Thus, it is remarkable that the ratio

of the second-order rate constants for alcoholysis by primary alcohols to hydrolysis of E-P falls in the range of 19−89 (Table 1). One may postulate that the extraordinary ability of water molecule to form hydrogen bonds to each other may render it less nucleophilic. For example, the abnormally low reactivity of hydroxide relative to alkoxide ion in aromatic nucleophilic substitution reactions at carbon atom was attributed to its high solvation (Murto, 1964). To our knowledge, there is no evidence that suggests that alcohols are better nucleophiles than water in nonenzymatic solution reactions involving phosphate monoesters. In fact, studies of solution reactions involving oxygen nucleophiles with acetyl phosphate (Herschlag & Jencks, 1989a) and phosphoanhydrides (Admiraal & Herschlag, 1995) suggest that water and alcohols have rather comparable reactivity toward phosphate.

In the PTPase-catalyzed reaction, the step that leads to the cysteinylphosphate enzyme intermediate (E-P) formation is accompanied by the expulsion of the leaving group (phenol or tyrosine). After the formation of E-P, the dephosphorylation event would occur by attack of water (or alcohol) that approaches from the just-vacated leaving group side of E-P with subsequent release of inorganic phosphate. It is most likely that the large selectivities for reactions of alcohols with E-P is due to the preferential hydrophobic interactions of alcohols with the binding pocket of the enzyme active site that holds the bulky leaving group portion of the substrates. This is in agreement with the increased effectiveness of the alcohol reactivity with longer aliphatic chain discussed above, requiring some sort of interaction between the enzyme and the nucleophile. The enhanced reactivity of alcohols toward E-P may be due to the more favorable positioning of the alcohols for effective nucleophilic attack at the expense of the extra binding energy they attained. It is noteworthy that if a binding site exists for alcohols, its affinities for these compounds are not very high since there is no kinetic evidence of saturation by alcohols up to 0.8 M concentration (Figure 1). Similar observations have been made in other enzyme-catalyzed partition reactions that involve alcohols, for example, with acyl-chymotrypsin (Inward & Jencks, 1965) and galactosyl-β-galactosidase (Richard et al., 1995). Alternatively, the preference for alcohols to react with E-P may result from perturbation of dielectric constant in the active site or change of activity coefficient of the nucleophile.

The Transition-State for the Dephosphorylation of E-P Is Highly Dissociative. Linear free energy relationships or Brønsted correlations are powerful probes of transition state structure in solution (Williams, 1984). In nonenzymatic solution reactions, the most striking characteristic of reactions of nucleophilic reagents with phosphate monoesters is the very small sensitivity of the rates to the basicity of the attacking nucleophilic reagent. For example, the $\beta_{nu:}$ value is 0.13 for *p*NPP dianion with amine nucleophile (Kirby & Jencks, 1965). The $\beta_{nu:}$ value for the reaction of the mixed anhydride acetyl phosphate with alcohols was found to be in the range of 0.10−0.14 (Herschlag & Jencks, 1989a). Recently, a $\beta_{nu:}$ value of 0.07 was obtained from studies of reactions of phosphoanhydrides with alcohols (Admiraal & Herschlag, 1995). These are consistent with the prevailing view in the literature that, in uncatalyzed solution reactions, phosphate monoesters hydrolyze via a dissociative mechanism involving an unsymmetrically "exploded" metaphosphate-like transition state where bond formation to the incoming nucleophile is minimal and bond breaking between phosphorus and the leaving group is substantial (Benkovic & Schray, 1978; Thatcher & Kluger, 1989; Cleland & Hengge, 1995).

Table 2: Second-Order Rate Constants for Stp1/Wt and Stp1/S18A[a]

β-substituted ethanol	pK_a	vol (Å^3)	Stp1 k_4 (M^{-1} s^{-1})	Stp1/S18A k_4 (M^{-1} s^{-1})
CF_3CH_2OH	12.37	74.52	0.56 ± 0.07	0.0051 ± 0.0001
$HC{\equiv}CCH_2OH$	13.55	74.29	0.74 ± 0.03	0.0072 ± 0.0006
$N{\equiv}CCH_2CH_2OH$	14.03	89.06	0.57 ± 0.08	0.0219 ± 0.0010
$ClCH_2CH_2OH$	14.31	82.44	0.71 ± 0.04	0.0240 ± 0.0007
$CH_3OCH_2CH_2OH$	14.82	99.27	0.76 ± 0.06	0.0261 ± 0.0009
$CH_2{=}CHCH_2OH$	15.52	95.77	1.88 ± 0.05	0.0494 ± 0.0010
CH_3CH_2OH	15.90	61.89	1.05 ± 0.03	0.0263 ± 0.0061
$CH_3CH_2CH_2OH$	16.10	81.84	2.19 ± 0.09	0.0530 ± 0.0018

[a] The reaction was performed in 50 mM succinate, 1 mM EDTA, pH 6.0, ionic strength = 0.15 M buffer at 30 °C. Molecular volumes of the alcohols were calculated with computer program Insight II. The pK_a values were obtained as described in the following references (Ballinger & Long, 1960; Murto, 1964; Takahashi et al., 1971).

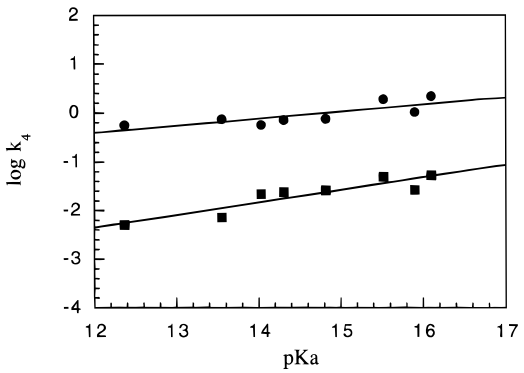


FIGURE 3: Brønsted plots of the second-order rate constant for the alcoholysis of E-P as a function of the basicity of the alcohol in the Stp1 (●) and Stp1/S18A (■) catalyzed reactions. Alcohols used are listed in Table 3.

To apply these physical organic chemistry principles to enzymology, one needs to be more careful in selecting compounds for a linear free energy relationship analysis, primarily due to the much increased steric complexity in an enzyme reaction system (Kirsch, 1972; Toney & Kirsch, 1989; Hollfelder & Herschlag, 1995). In the current study, we have shown that the rate of Stp1-catalyzed phosphoryl transfer from E-P to alcohols is sensitive to substitutions at the α carbon and increases as the molecular volume of the alcohols expand, even when the intrinsic reactivity is kept constant. In order to minimize the influence of steric effects and hydrophobic interactions on the transfer reactions, we have chosen eight β-substituted ethanols that are all primary alcohols and have similar molecular volumes (Table 2). The alcohols selected cover a pK_a range of 12.37−16.1 (Ballinger & Long, 1960; Murto, 1964; Takahashi et al., 1971).

To probe the transition state of the dephosphorylation step, we have studied the effect of changing pK_a on the second-order rate constants for the reaction of β-substituted ethanols with E-P. The second-order rate constants for alcoholysis of E-P are listed in Table 2. A Brønsted plot of log k_4 against the pK_a of the attacking alcohol gives a slope of $\beta_{nu:}$ = 0.14 ± 0.04 (r = 0.81) for the wild-type Stp1-catalyzed reaction (Figure 3). In an attempt to correct for the effect due to slight variation of molecular volume among the β-substituted ethanols, we also plotted [log k_4 − V(molecular volume)] versus the pK_a of alcohols (Toney & Kirsch, 1989) (data not shown). This correction does not improve the correla-

tion, and neither does it lead to any changes in the slope (the corrected Brønsted coefficient $\beta_{nu:}' = 0.14 \pm 0.04$; $r = 0.81$). Figure 3 therefore reflects purely the dependence of phosphoryl transfer from E-P to alcohols on the intrinsic chemical reactivity of the nucleophiles.[2] The important conclusion from the data (Figure 3 and Table 2) is that the nucleophilic reactivity of alcohols toward E-P is not significantly influenced by the basicity of the nucleophiles. By reference to model studies cited above, the small dependence of the second-order rate constant (k_4) on basicity ($\beta_{nu:} = 0.14$) for the reactions of alcohols with E-P suggests that the transition state for the dephosphorylation step in the Stp1-catalyzed reaction is also highly dissociative. Interestingly, it was previously shown that μ-monothiopyrophosphate undergoes hydrolysis through a discrete metaphosphate intermediate without nucleophilic participation (Lightcap & Frey, 1992). The fact that the rate of phosphoryl transfer from the cysteinyl phosphate intermediate to alcohols depends on the nucleophile basicity indicates that the entering nucleophile is a required participant in the transition state and argues against the existence of a free metaphosphate intermediate in the PTPase-catalyzed reaction.

The slope of a Brønsted plot or the β value is often interpreted as a measure of the amount of charge development or bond formation in the transition state. The correct interpretation of $\beta_{nu:}$ in terms of the extent of bond formation in the transition state between the nucleophile and the phosphorus depends on the proper choice of a standard equilibrium for comparison. Since we are correlating the kinetics of a phosphoryl transfer reaction with the equilibrium process of proton transfer (i.e., pK_a), it is essential to recalibrate the parameter with the equilibrium phosphoryl transfer. The β_{EQ} value for the complete equilibrium transfer of the phosphoryl group from phosphate monoester dianions is −1.35 (Bourne & Williams, 1984). The extent of bond formation is given by $\beta_{nu:}/\beta_{EQ}$ (Williams, 1992). Thus, it appears that, in the nucleophilic reaction of E-P with alcohol, only 0.10 of a positive charge has developed on the alcoholic oxygen atom in the transition state. This indicates a minimal amount of bond formation (10%) between the nucleophile and the phosphorus by the time the transition state is reached.[3] It follows that the less the extent of bond formation in the transition state, the less sensitive the reaction will be to the electron density on the incoming nucleophile.

Recently, we have studied the nature of the transition state of the *Yersinia* PTPase and the mammalian PTP1-catalyzed hydrolysis of *p*NPP by measuring the heavy-atom kinetic isotope effects (Hengge et al., 1995). This technique is a competitive method which measures effects through the first irreversible step of the mechanism, shown as k_2 in Scheme 1. The transition state for the phosphorylation step (i.e. E-P formation) is similar for both enzymes and is highly dissociative and similar to that in solution (Hengge et al., 1995). Preliminary kinetic isotope effect experiments indicate that the transition state for E-P formation is also dissociative in the Stp1-catalyzed *p*NPP hydrolysis (A. C. Hengge & Z.-Y. Zhang, unpublished result). It is thus concluded that for both chemical steps (i.e., E-P formation and breakdown) in the PTPase-catalyzed reaction, the transition state structure is highly dissociative and resembles that for the uncatalyzed solution reaction.

Ser18 in the Active Site of Stp1 Plays an Important Role in E-P Hydrolysis. All PTPases, including the dual specificity phosphatases and the low M_r PTPases, contain a unique and conserved active site sequence (H/V)**C**(X)$_5$**R**(S/T) called the PTPase signature motif (Zhang et al., 1994b). Mechanistic studies have demonstrated that the invariant Cys residue is essential for phosphatase activity and formation of E-P (Zhang, 1990; Guan & Dixon, 1991; Wo et al., 1992; Cho et al., 1992; Zhou et al., 1994), whereas the invariant Arg residue in the signature motif plays an important role in substrate binding and transition state stabilization (Zhang, Z.-Y., et al., 1994b). In addition to the essential Cys and Arg residues, a conserved Ser or Thr can also be found in the PTPase signature motif immediately after the invariant Arg residue (Zhang et al., 1995b). In the bovine low M_r PTPase structure (Su et al., 1994; Zhang, M., et al., 1994) as well as in the *Yersinia* PTPase (Stuckey et al., 1994) and the human PTP1B (Barford et al., 1994) structures, the hydroxyl group of the conserved Ser/Thr is approximately 3 Å to the Sγ of the active site Cys residue (Figure 4), making a reasonably good S−HO hydrogen bond (Gregoret et al., 1991). Site-directed mutagenesis and kinetic experiments have suggested that the hydroxyl group in the PTPase signature motif plays a critical role in E-P hydrolysis in the *Yersinia* PTPase (Zhang et al., 1995b) and the dual specificity phosphatase, VHR (Denu & Dixon, 1995). Although the tyrosine-specific PTPases, the dual specificity phosphatases, and the low M_r phosphatases share the active site motif (H/V)**C**(X)$_5$**R**(S/T), they display little amino acid sequence identity outside of the active site. It is not clear whether a similar role can be proposed for the conserved hydroxyl group in Stp1.

[2] A reviewer raised the question of whether the $\beta_{nu:}$ value could be complicated by the involvement of general base Asp128 in the transition state and solvation effects on nucleophilicity. The small $\beta_{nu:}$ value could be accounted for by the general base assisted partial proton removal in the transition state during the nucleophilic attack, because substituent effects on the nucleophilicity and on the ease of deprotonation are opposite in direction and may cancel one another out. We have shown previously that replacement of Asp128 with a Glu residue results in a weaker general base at this position (Wu & Zhang, 1996). One would predict that the $\beta_{nu:}$ value for the D128E mutant would be larger than that of the wild type, if proton removal is significant in the transition state. We found that a Brønsted plot of log k_4 against the pK_a of the attacking alcohol gives a slope of $\beta_{nu:} = 0.11 \pm 0.08$ for the Stp1/D128E-catalyzed reaction. Thus the $\beta_{nu:}$ value for the D128E mutant is not significantly different from that of the wild type (0.14 ± 0.04). This suggests that the extent of deprotonation of the nucleophile by the general base is probably marginal in the transition state and exerts little influence on the magnitude of the observed $\beta_{nu:}$ value. This is consistent with the transition state being highly dissociative so that there is minimal bond formation between the nucleophile and the phosphorus and minimal nucleophile activation (deprotonation) is required in the transition state. As for solvation effects on nucleophilicity, there is currently no good way to quantify the effects of solvation on the $\beta_{nu:}$ value. Solvation effects generally decrease the observed $\beta_{nu:}$ value, especially for nitrogen based nucleophiles. For example, a negative value of $\beta_{nu:}$ has been observed for phosphoryl transfer to amines which is attributed to solvation (Jencks et al., 1986). Similar solvation effects on phosphoryl transfer to alcohols have not been reported. Since the active site of low M_r PTPase is situated at an open cleft and is relatively exposed to solvent (Zhang, M., et al., 1994), one would expect that solvation effects on the enzymic reaction to be similar to those in uncatalyzed solution reaction. The transition state of the solution reactions have been well characterized by a variety of other means and serve as an appropriate standard in the present study. Thus conclusions based on comparisons of $\beta_{nu:}$ values from Stp1-catalyzed reactions with those for the uncatalyzed solution reactions are reasonable.

[3] It should be noted that since the β_{EQ} value for phosphate thioester has not been determined, the calculated extent of bond formation between the nucleophile and the phosphorus in the transition state can only be viewed as approximate.

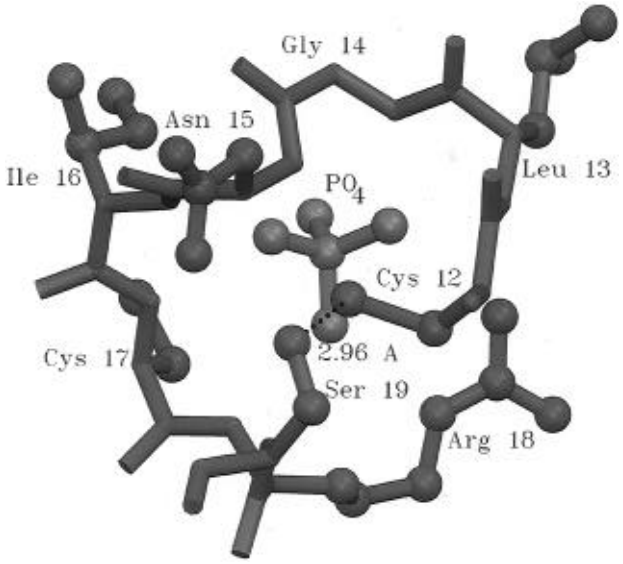


FIGURE 4: Active site conformation of the PTPase signature motif corresponding to residues Cys12 to Ser19 in the bovine low M_r PTPase (Zhang, M., et al., 1994). The hydrogen bond between the sulfur atom of Cys12 and the hydroxyl group of Ser19 is highlighted.

In Stp1, the corresponding hydroxyl group in the PTPase signature motif is contained in Ser18. To evaluate the role of Ser18 in catalysis, it was changed to alanine, which lacks the hydroxyl group of the Ser side chain. The S18A mutant was purified to homogeneity using procedures similar to those described for the wild-type Stp1 (Zhang et al., 1995a). S18A had chromatographic and spectral characteristics similar to those of the wild type suggesting that there were no major changes in the structure of the mutant. In addition, the affinity of S18A for phosphate, a competitive inhibitor of the enzyme, is 1.03 ± 0.10 mM at pH 6, which is similar to that of the wild type enzyme (2.4 ± 0.3 mM) suggesting that there is no significant alteration in the active site conformation. At pH 6.0 and 30 °C (I = 0.15 M), the k_{cat} and K_m values of the S18A mutant Stp1-catalyzed hydrolysis of *p*NPP are 0.112 ± 0.004 s^{-1} and 0.016 ± 0.002 mM, while the k_{cat} and K_m values for the wild-type Stp1 are 3.00 ± 0.09 s^{-1} and 0.040 ± 0.004 mM. Thus, S18A exhibits k_{cat} and k_{cat}/K_m values that are 27- and 11-fold lower than those of the wild-type enzyme, respectively.

To further evaluate the specific contribution of the active site hydroxyl group at Ser18 to the individual steps of Stp1 catalysis, burst kinetic characterization of the S18A was performed. Unfortunately, S18A at high protein concentrations (10−60 μM) gradually precipitated out of solution during the course of the stopped-flow experiments at pH 6.0, 50 mM succinate, 1 mM EDTA, I = 0.15 M buffer and 30 °C. High concentrations of S18A could be maintained soluble if the ionic strength of the buffer was raised to 1.0 M by adding NaCl. All of the subsequent pre-steady-state experiments were therefore performed at pH 6.0, 50 mM succinate, 1 mM EDTA, I = 1.0 M buffer and 30 °C (Figure 5). The k_{cat} and K_m values of the S18A-catalyzed hydrolysis of *p*NPP in this buffer are 0.092 ± 0.001 s^{-1} and 0.090 ± 0.004 mM, while the k_{cat} and K_m values for the wild-type Stp1 are 2.66 ± 0.02 s^{-1} and 0.96 ± 0.04 mM. Thus, the increase in salt concentration has only a marginal effect on k_{cat}, whereas marked increases in the apparent K_m value are noticed for both the wild type and the S18A mutant. Figure 5 shows the differential effects of Ala substitution at residue

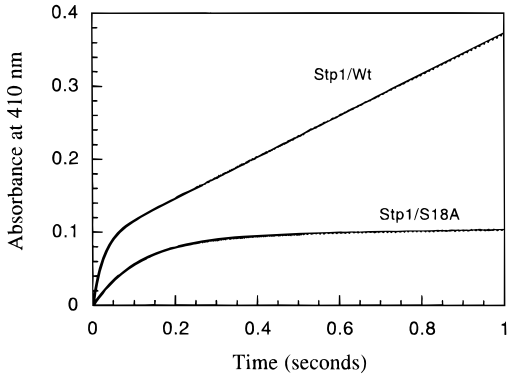


FIGURE 5: Burst kinetics of the wild-type and S18A mutant Stp1 with *p*NPP as a substrate at pH 6 and 30 °C in 50 mM succinate, 1 mM EDTA, and I = 1.0 M buffer. The concentrations of the wild-type and S18A mutant were 56.7 and 59.5 μM, respectively. The final *p*NPP concentration was 10 mM. Each stopped-flow trace was an average of at least eight individual experiments. The solid line represents a theoretical fit of the data to the equation [*p*-nitrophenolate] = $At + B(1 - e^{-bt}) + C$.

Table 3: Pre-Steady-State Kinetic Parameters of Stp1/wt and Stp1/S18A[a]

	k_{cat} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})
Stp1/Wt	2.50	37.2	2.68
Stp1/S18A	0.074	8.59	0.075

[a] All of the stopped-flow experiments were performed at pH 6.0 and 30 °C in 50 mM succinate, 1 mM EDTA, and I = 1.0 M buffer.

Ser18 on k_2 and k_3 at pH 6.0. Table 3 compares the pre-steady-state kinetic parameters for both the wild type and the S18A mutant Stp1-catalyzed hydrolysis of *p*NPP. The k_{cat} values determined from the burst kinetic experiments (Table 3) are comparable to those measured from steady-state experiments. Elimination of the hydroxyl group at Ser18 decreases the rate of E-P formation (k_2) and breakdown (k_3) by 4.3- and 35.7-fold, respectively. Similarly, substitution of the corresponding Thr residue by an Ala in the *Yersinia* PTPase resulted in a 2.4- and 18.8-fold reduction in k_2 and k_3, respectively, at pH 6 (Zhang et al., 1995b). Thus, in agreement with the *Yersinia* PTPase (Zhang et al., 1995b) and the dual specificity phosphatase VHR (Denu & Dixon, 1995), the main function for the conserved hydroxyl group in Stp1 is to facilitate the breakdown of the phosphoenzyme intermediate. How this is accomplished by the hydroxyl side chain has remained conjectural. It is also not clear why disruption of the hydroxyl−thiolate interaction causes differential effects on the two chemical steps.

The Conserved Hydroxyl Group in the PTPase Signature Motif Promotes and Stabilizes the Dissociative Transition State in the Dephosphorylation Reaction. An important strategy to promote a dissociative mechanism is to stabilize the buildup of negative charge on the leaving group (Benkovic & Schray, 1978; Herschlag & Jencks, 1990). The dissociative transition state for E-P formation in the PTPase reaction is stabilized by an active site Asp residue which facilitates the departure of the leaving phenoxide (Hengge et al., 1995; Zhang et al., 1995c; Wu & Zhang, 1996). It has been hypothesized that if the transition state for the E-P dephosphorylation step is also dissociative, charge stabilization on the leaving thiolate will be important since P−S bond breaking is substantial in the transition state (Zhang et al., 1995b). This charge stabilization can be effected by a hydrogen bond between the sulfur atom of the active site

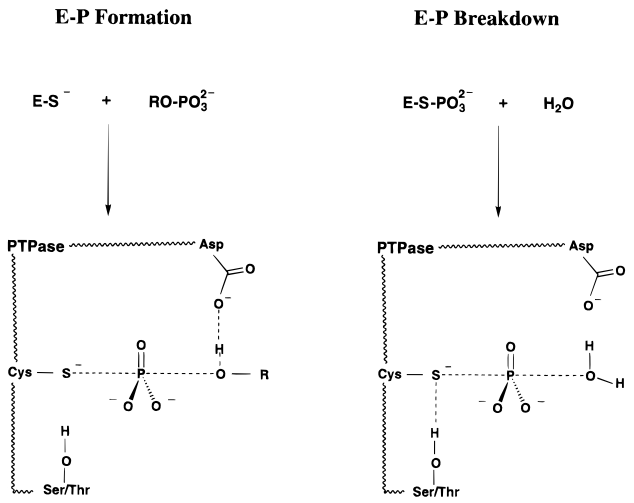


FIGURE 6: Suggested transition state structures for the phosphorylation and dephosphorylation steps in the PTPase-catalyzed reaction. In the E-P formation step, the proton is shown predominantly on the side chain the Ser/Thr residue, while in the E-P breakdown step, the proton is shown predominantly on the attacking water molecule, as expected from the nature of the transition states.

Cys residue and the hydroxyl group. The loss of such an interaction would make the phosphocysteine more resistant to breakdown. In essence, the conserved hydroxyl group in the PTPase signature motif provides substantial driving force for the thiophosphate enzyme intermediate to go through a dissociative pathway. We have shown in this study that the transition state for E-P hydrolysis is indeed dissociative. The suggested transition state structures and the likely mechanisms for their stabilization in the PTPase-catalyzed E-P formation and breakdown steps are depicted in Figure 6. Since there is very little bond formation between the phosphorus and the attacking nucleophile in a dissociative transition state, minimal activation is required for the nucleophilic attack (Figure 6). Thus, it is understandable that elimination of the hydroxyl group at position 18 has a minimal effect on the phosphorylation step, because little nucleophilic activation is needed for the reaction. We would also predict that the elimination of the active site hydroxyl group would make the dephosphorylation reaction *less* dissociative.

To validate this supposition, we have determined the $\beta_{nu:}$ value for the S18A mutant Stp1-catalyzed phosphoryl transfer to alcohols under exactly the same experimental conditions used for the wild-type enzyme. The S18A mutant displays similar sensitivity to steric hindrance (data not shown) and molecular volume of alcohols (Figure 2) as the wild-type enzyme, indicating again that there is no significant alteration in the active site. The Brønsted plot of the second-order rate constant for the alcoholysis (k_4) of E-P as a function of the basicity of the alcohol in the S18A-catalyzed reaction is shown in Figure 3, which gives a slope of $\beta_{nu:} = 0.26 \pm 0.05$ ($r = 0.91$). Again, correction for the slight variation of molecular volume among the β-substituted ethanols ([log $k_4 - V$(molecular volume)] versus the pK_a of alcohols) does not improve the correlation significantly, and neither does it lead to any significant changes in the slope (the corrected Brønsted coefficient $\beta_{nu:}' = 0.24 \pm 0.03$; $r = 0.94$). It appears that basicity alone adequately predicts the rate constants. Thus, the fact that the $\beta_{nu:}$ for S18A is almost twice that of the wild type, which indicates almost twice as much of a positive charge has developed on the alcoholic oxygen atom in the transition state, lends support to the prediction that the transition state of the S18A-catalyzed E-P dephosphorylation reaction has become *less* dissociative in comparison with that of the wild-type enzyme.

In contrast to phosphate monoesters, phosphate triesters react with associative transtion states (Benkovic & Schray, 1978; Cleland & Hengge, 1995). It is noteworthy that $\beta_{nu:}$ values for reactions of oxyanions with phosphate triesters range from 0.30 to 0.48, depending on the leaving groups (Khan & Kirby, 1970). It is thus conceivable that the transition state for the S18A-catalyzed reaction contains substantial associative character.

Mechanistic Implications. Nonenzymatic nucleophilic displacement reactions on phosphate monoesters are believed to involve an "exploded" metaphosphate-like dissociative transition state (Thatcher & Kluger, 1989; Herschlag & Jencks, 1989a; Hengge et al., 1994; Cleland & Hengge, 1995). A prerequisite for the understanding of enzyme-catalyzed phosphoryl transfer reactions is the elucidation of the nature of the enzymic transition state. What is the preferred mechanistic route for an enzyme-catalyzed phosphoryl transfer reactions? Do enzymes catalyze phosphoryl transfers via a dissociative or an associative mechanism? Different strategies may be utilized by an enzyme to accelerate a reaction depending on the nature of the transition state.

An associative mechanism had been favored for enzyme-catalyzed phosphoryl transfer reactions since an enzyme could stabilize the increased negative charge on the phosphoryl moiety in the transition state (Hasset et al., 1982; Mildvan & Fry, 1987; Cullis, 1987). It has been argued that general acid catalysis to accelerate the departure of leaving group should be enzymatically important regardless of the mechanism of phosphorylation (i.e., associative or dissociative) and that there should be little requirement for general base catalysis in dissociative mechanism (Benkovic & Schray, 1978). In order to maximize the catalytic power from proton transfer to a general base at the active site, it is conceivable that the enzyme may select a somewhat more associative transition state with more bond formation to the entering nucleophile (Herschlag & Jencks, 1989b). However, comparisons of the heavy atom kinetic isotope effects on the PTPase-catalyzed *p*NPP hydrolysis (Hengge et al., 1995; A. C. Hengge, and Z.-Y. Zhang, unpublished result) and the $\beta_{nu:}$ value of the Stp1-catalyzed dephosphorylation of E-P (this work) with the solution data reveal that the transition state structures of both the PTPase-catalyzed E-P formation and breakdown steps are highly dissociative and similar to that in the uncatalyzed reaction. Recent evidence suggests that the transition states for other phosphoryl transfer enzymes are also dissociative. For example, the slightly inverse secondary ^{18}O isotope effects with both alkaline phosphatase (Weiss & Cleland, 1989) and yeast hexokinase (Jones et al., 1991) are consistent with a dissociative transition state for these enzymic reactions. The large negative $\beta_{lg} = -0.8$ obtained from a linear free energy relationship analysis for cleavage of aryl phosphorothioates catalyzed by alkaline phosphatase also suggests that the transition state remains predominately dissociative in character (Hollfelder & Herschlag, 1995). It remains to be seen if a dissociative mechanism is chosen by all phosphoryl transfer enzymes.

In summary, we have shown in this paper that the low M_r PTPase Stp1 catalyzes phosphoryl transfer reactions from E-P to alcohols. This reaction is sterically sensitive to groups adjacent to the attacking nucleophilic atom and to binding interactions away from the reaction center. A linear free

energy analysis of the effect of basicity of a series of β-substituted ethanols on the reactivity of these nucleophiles toward E-P reveals a transition state with considerable dissociative character for the dephosphorylation step. We show that the conserved hydroxyl group in the Stp1 active site is important for E-P hydrolysis. We further demonstrated that elimination of the hydroxyl group renders the transition state *less* dissociative for the S18A mutant, providing a clear example in which substitution of a single residue in the active site causes a substantial alteration in the nature of the transition state. Thus, the main function of the conserved hydroxyl group is to stabilize the dissociative transition state. Although there is no significant sequence identity among the PTPases, the dual specificity phosphatases and the low M_r PTPases, based on the available biochemical and structural data, it is likely that they all utilize the same structural feature, the PTPase signature motif, within their active sites and employ a similar strategy for phosphate monoester hydrolysis.

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