Probing the Function of Asp128 in the Low Molecular Weight Protein-Tyrosine Phosphatase-Catalyzed Reaction. A Pre-Steady-State and Steady-State Kinetic Investigation

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ABSTRACT: The role of Asp128 in the catalytic mechanism of the low M_r protein-tyrosine phosphatase (PTPase) from the fission yeast *Schizosaccharomyces pombe* has been investigated by a combination of site-directed mutagenesis and pre-steady-state and steady-state kinetic analysis. The corresponding aspartic acid in the bovine enzyme is located on a loop adjacent to the phosphate-binding loop and forms a hydrogen bond with the oxygen atom of the bound sulfate or phosphate that is structurally homologous to the ester oxygen in substrates [Su et al. (1994) *Nature 370*, 575−578; Zhang, M., et al. (1994) *Biochemistry 33*, 11097−11105]. Asp128 has been replaced by a Glu, an Asn, and an Ala. The k_{cat} for the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) decreases by factors of 6.7, 400, and 650 for the mutants D128E, D128N, and D128A, respectively. Compared to the wild type, the binding affinity for phosphate is decreased 2 and 4.3-fold, respectively, for the D128A and D128N mutants, whereas no change in affinity is observed for the D128E mutant. An evaluation of the burst kinetics demonstrates that Asp128 plays a role in both the phosphoenzyme intermediate formation (k_2) and breakdown (k_3). Thus, substitution at Asp128 by a Glu, an Asn, or an Ala reduces k_2 by 17, 7480, and 11900 and reduces k_3 by 6.2, 380, and 40. The greater effect on k_2 than k_3 is consistent with a dissociative transition-state for the low M_r PTPase-catalyzed reaction. Results from rapid kinetics, partition experiments, and leaving group dependence experiments suggest that for the wild type and D128E mutant, the rate-limiting step is k_3, whereas k_2 has become rate-limiting for the D128N mutant. With the exception of *p*NPP, k_2 may also be rate-limiting for D128A. Taken together, these results are consistent with Asp128 or Glu128 acting as a general acid to donate a proton to the phenolate leaving group in the phosphorylation step, and the carboxylate side chain plays a role as a general base to activate a nucleophilic water molecule in the dephosphorylation step. The presence of the general acid ensures productive partitioning toward phosphoenzyme formation. In the absence of the general acid, the nature of the transition-state for the phosphorylation step is sensitive to the pK_a of the attacking active site thiol group and changes with the structure of the leaving group.

INTRODUCTION

There is growing appreciation of the biological importance of protein-tyrosine phosphatases (PTPases) in signal transduction (Hunter, 1995). The PTPases constitute a family of enzymes (now >50 members) that rival the protein-tyrosine kinases in terms of structural diversity and complexity. Unlike protein kinases, where tyrosine specific and serine/threonine specific kinases share sequence identity, the PTPases show no sequence similarity with serine/threonine phosphatases or the broad specificity phosphatases such as acid or alkaline phosphatases. The unique feature that defines the PTPase family is the active site sequence (H/V)**C**(X)$_5$**R**(S/T) called the PTPase signature motif in the catalytic domain (Cirri et al., 1993; Zhang et al., 1994a).

Interestingly, the PTPase signature motif can also be found in the structures of two additional groups of enzymes that can bring about phosphomonoester hydrolysis, namely, the VH1-like dual-specificity phosphatases (Guan et al., 1991), and the low molecular weight (18 kDa) PTPases (Zhang & Van Etten, 1990). The low M_r PTPases, whose biological function is unknown, were previously found only to exist in mammalian species. Genetic studies of fission yeast (*Schizosaccharomyces pombe*) with temperature sensitive mutations of *cdc25* (*i.e., cdc25-22*) have identified a gene that when overexpressed, rescues *cdc25-22* (Mondesert et al., 1994). This gene, named *stp1*$^+$ (*s*mall *t*yrosine *p*hosphatase), encodes a protein that is highly similar (42% identical) to the mammalian low M_r PTPases. We have recently shown that low M_r PTPases can dephosphorylate both phosphotyrosyl and phosphoseryl/threonyl protein substrates (Zhang et al., 1995). These findings suggest that low M_r PTPases may represent another group of dual specificity phosphatases that may be involved in cell cycle control.

The low M_r and the VH1-like phosphatases display little amino acid sequence identity with the classical PTPases. The only similarities among these three groups of phosphatases are the relative placements of the essential cysteine and arginine residues in the active sites that constitute the PTPase signature motif (H/V)**C**(X)$_5$**R**(S/T). Although the bovine low M_r PTPase has distinct topologies compared with those of the PTP1B and the *Yersinia* PTPase, residues of the PTPase signature motif (12−19 in the low M_r PTPase, 403−410 in the *Yersinia* PTPase, and 214−222 in PTP1B) form a similar

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loop structure termed the phosphate-binding loop between the β-turn at the COOH-terminus of a β strand and the first turn of an α helix (Su et al., 1994; Zhang, M., et al., 1994; Barford et al., 1994; Stuckey et al., 1994). These different phosphatase structures are striking examples of convergent evolution achieving highly similar active site clefts, and the similarities in the conserved active site motifs may suggest a common mechanism to bring about phosphate monoester hydrolysis in these otherwise very different molecules. Indeed, the invariant Cys residue has been shown to be essential for phosphatase activity and formation of a covalent cysteinyl phosphoenzyme intermediate (Zhang, 1990; Guan & Dixon, 1991; Wo et al., 1992; Cho et al., 1992), whereas the invariant Arg residue in the signature motif has been shown to play an important role in substrate binding and transition-state stabilization (Zhang et al., 1994a).

In addition to nucleophilic catalysis and transition-state stabilization, it appears that all three groups of phosphatases also utilize general acid/base to facilitate the catalytic turnover (Zhang et al., 1994b). 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 a dissociative mechanism (Benkovic & Schray, 1978). By site-directed mutagenesis and pH kinetic analysis, Asp356 in the *Yersinia* PTPase was first demonstrated to be the general acid in the PTPase-catalyzed reaction (Zhang et al., 1994b). Subsequently, Asp92 in the dual specificity phosphatase VHR was shown to play a similar role (Denu et al., 1995). The crystal structures of the bovine low M_r enzyme have also implicated an aspartic acid, Asp129, on a loop adjacent to the phosphate-binding loop as a potential catalytic general acid (Su et al., 1994; Zhang, M., et al., 1994). Recent mutational studies provided evidence for the importance of Asp129 in catalysis, but the detailed mechanism and the specific step(s) that is effected by Asp129 remained controversial due to the inherent limitations of steady-state kinetic analysis (Taddei et al., 1994; Zhang, Z., et al., 1994).

We have recently described an efficient procedure for obtaining large quantities of the yeast low M_r PTPase Stp1 (Zhang et al., 1995). Since the turnover number for the Stp1-catalyzed reaction is 6 times slower than that of the mammalian low M_r PTPases, burst kinetic analysis is possible at more physiological temperatures. This has enabled us to determine the individual rate constants directly associated with the formation (k_2) and breakdown (k_3) of the phosphoenzyme intermediate. The yeast enzyme is thus a good model for detailed rapid kinetic studies in order to define the function of essential active site residues involved in catalysis. Detailed pre-steady-state and steady-state kinetic analysis of the corresponding mutants of Asp128 in the yeast enzyme should lead to a better understanding of the role of this critical aspartic acid in catalysis. This paper describes such a study.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl phosphate (*p*NPP), β-naphthyl phosphate, 4-methylumbelliferyl phosphate, and phenyl phosphate were purchased from Sigma. Aryl phosphate monoesters, 4-acetylphenyl, 4-cyanophenyl, 3-nitrophenyl, 3-chlorophenyl, 4-(trifluoromethyl)phenyl, 4-ethylphenyl, 4-fluorophenyl, 4-chlorophenyl, and 4-methylphenyl phosphate, were synthesized as described (Zhang & Van Etten, 1991a). Solutions were prepared using deionized and distilled water.

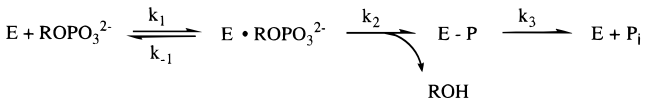
Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using the Muta-Gene *in vitro* mutagenesis kit from Bio-Rad. The plasmid pUC118-Stp1 (Zhang et al., 1995) encoding the yeast low M_r PTPase, Stp1 (small *t*yrosine *p*hosphatase), was used to make single-stranded DNA for site-directed mutagenesis. The oligonucleotide primers used for the desired substitutions were as follows: D128A, TCGTTGATGCCCCTTATT; D128N, ATCGTTGATAACCCTTAT; and D128E, CGTTGATGAGCCTTATTA. The underlined bases indicate the change from the naturally occurring nucleotides. All of the mutations were verified by DNA sequencing.

Expression and Purification of the Recombinant Enzymes. The wild type Stp1 and all of the mutant proteins were expressed in *Escherichia coli* and purified to homogeneity as described previously (Zhang et al., 1995).

Enzyme Assay. Since physiological substrates are not known for PTPases, and specific stoichiometrically phosphorylated protein substrates are difficult to obtain in large quantities sufficient for detailed kinetic analysis, artificial substrates such as *p*NPP are currently utilized for mechanistic studies. Furthermore, the optimal k_{cat} values for *p*NPP and phosphotyrosine-containing peptide substrates are similar for PTPases, suggesting that mechanistic studies using *p*NPP as a substrate should be relevant to physiological substrates. The PTPase activity of Stp1 was assayed with various concentrations of substrates at 30 °C in pH 6.0, 50 mM succinate buffer. The buffer contained 1 mM EDTA, and the ionic strength of the solution was kept at 0.15 M using NaCl. For *p*NPP, the reaction was initiated by addition of enzyme and quenched after 2−3 min by addition of 1 mL of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18 000 M^{-1} cm^{-1}. For nonchromogenic substrates, enzyme activity was determined by measuring the production of inorganic phosphate (Zhang & Van Etten, 1990). Michaelis−Menten kinetic parameters were determined from a direct fit of the v vs [S] data to the Michaelis-Menten equation using the nonlinear regression program GraFit (Erithacus Software).

Rapid Kinetics. Pre-steady-state kinetic measurements of the Stp1-catalyzed hydrolysis of *p*NPP were conducted using a Hi-Tech SF-61 stopped-flow spectrophotometer (dead time, 2 ms) with an observation cell length of 1.0 cm. Fast reactions at 30 °C were monitored by the increase in absorbance at 410 nm of the *p*-nitrophenolate product. The extinction coefficient for *p*-nitrophenolate is 1325 M^{-1} cm^{-1} at pH 6.0 and 30 °C. The buffer used was 50 mM succinate, 1 mM EDTA, $I = 0.15$ M, pH 6.0. The analysis of burst kinetics for Stp1-catalyzed hydrolysis of *p*NPP has been described (Zhang et al., 1995). Briefly, when [S] ≫ [E], the increase in *p*-nitrophenolate concentration as a function of time can be described as [*p*-nitrophenolate] $= At + B(1 - e^{-bt})$. The individual rate constants for the enzyme phosphorylation (k_2) and dephosphorylation (k_3) can be determined from the exponential ($b = k_2 + k_3$) and the linear

Scheme 1



phase ($A = k_2k_3/(k_2 + k_3)$), respectively (Scheme 1). The size of the burst $B = E_o[k_2/(k_2 + k_3)]^2/(1 + K_m/S_o)^2$ and is proportional to the active enzyme concentration. So if k_2 and k_3 are comparable, one should observe a "burst" of *p*-nitrophenol production using *p*NPP as a substrate. Data collection and analysis were carried out as described previously (Zhang, 1995).

Kinetics and pH Dependence of Iodoacetate Inactivation of Stp1. The experimental procedures for following the inactivation of Stp1 and the analysis of the pH dependence of the inactivation rate for the determination of the active site thiol pK_a were the same as described earlier (Zhang & Dixon, 1993).

RESULTS AND DISCUSSION

The low M_r PTPases effect catalysis of phosphate monoester hydrolysis through the formation of a covalent thiophosphate enzyme intermediate involving the active site cysteine residue as a nucleophile (Zhang, 1990; Wo et al., 1992). The minimal kinetic scheme for the catalyzed reaction is given in Scheme 1, which is composed of substrate binding, followed by two chemical steps, phosphorylation (k_2) and dephosphorylation (k_3) of the enzyme, where E is the enzyme, $ROPO_3^{2-}$) the substrate, E•$ROPO_3^{2-}$ the enzyme−substrate Michaelis complex, E−P the phosphoenzyme intermediate, ROH the phenol or alcohol, and P_i inorganic phosphate. For both the bovine and the yeast enzyme, the decomposition of the cysteinyl phosphate enzyme intermediate (k_3) is the rate-limiting step for the overall hydrolysis reaction (Zhang & Van Etten, 1991a; Zhang et al., 1995). The step leading to cysteinyl phosphate formation is presumably facilitated by the protonation of the ester oxygen atom in the leaving group. This is required for stabilization of the trigonal bipyramidal transition-state toward loss of the oxygen of the leaving group rather than the sulfur of the cysteinyl nucleophile. Indeed, the three-dimensional structures of the bovine enzymes isolated from liver and heart have allowed the identification of the possible candidate for the proton donor (Su et al., 1994; Zhang, M., et al., 1994). Both structures reveal that Asp129, located on a loop adjacent to the phosphate-binding loop but opposite the nucleophilic cysteine, is pointed toward the bound sulfate and phosphate, respectively. In one structure, solved at pH 5.5 with a bound sulfate at the active site (Su et al., 1994), the side chain of Asp129 is shown to form a hydrogen bond with one oxygen of the sulfate anion. The distance between the carboxylate oxygen and the sulfate oxygen is 2.7 Å. This suggests that the side chain of Asp129 is protonated in this condition. In the other structure, determined at pH 7.5 with a phosphate anion bound at the active site (Zhang, M., et al., 1994), the side chain of Asp129 is 3.66 Å away from the phosphate. This increase in distance could be caused by charge repulsion between the phosphate and the carboxylate, since at pH 7.5 the carboxyl group of Asp129 is likely deprotonated. Since the oxygen atom in the bound oxyanion that interacts with Asp129 is structurally homologous to the scissile oxygen of a phosphate monoester substrate, one role proposed for Asp129 is to donate a proton to the leaving group during the phosphoenzyme formation (Su et al., 1994; Zhang, M., et al., 1994). After the formation of the phosphoenzyme intermediate, the dephosphorylation event would occur by attack of water that approaches from the just-vacated leaving group side on the phosphoenzyme intermediate with subsequent release of inorganic phosphate. It is thus conceivable that Asp129 could function in the second step by activating a water molecule for the hydrolysis of the phosphoenzyme intermediate (Su et al., 1994).

Although the importance of Asp129 in catalysis has been confirmed using techniques of site-directed mutagenesis (Taddei et al., 1994; Zhang, Z., et al., 1994), the detailed mechanism and the specific step(s) that is effected by Asp129 requires further investigation. In both cases, Asp129 was changed to an Ala residue. However, the conclusions from the two papers differ drastically. For example, in one study, Asp129 is concluded to be the proton donor to the leaving group in the phosphorylation step, and its mutation to alanine results in a change in the rate-limiting step of the catalysis from dephosphorylation (k_3) to phosphorylation (k_2) (Zhang, Z., et al., 1994). In the other study, Asp129 is suggested to be involved in both steps of the catalytic mechanism and the rate-limiting step of the D129A mutant is reported to still correspond to k_3 (Taddei et al., 1994). These contradictory results are likely caused by the inherent limitations of steady-state kinetic analysis.

Pre-steady-state burst kinetic experiments have been used to detect intermediates on reaction pathways, to measure their rates of formation and decay, and to quantitate enzyme active site concentrations (Fersht, 1985). Because the enzyme is used in relatively large amounts and the events involving changes on the enzyme are in effect observed directly, these type of experiments appear more definitive. Using *p*NPP as a substrate, we have demonstrated burst kinetics at 30 °C and pH 6.0 with Stp1 (Zhang et al., 1995). This permitted the determination of rate constants directly associated with the formation (k_2) and breakdown (k_3) of the phosphoenzyme intermediate. Thus, an evaluation of the burst kinetics combined with the technique of site-directed mutagenesis should allow us to ascertain specific contributions of active site residues to the individual steps of the low M_r phosphatase-catalyzed reaction. The corresponding putative general acid in Stp1 is Asp128 (Zhang et al., 1995). Detailed pre-steady-state and steady-state kinetic analysis of the site-directed mutants of Asp128 in the yeast enzyme should resolve previous reported discrepancies and lead to a better understanding of the role of this critical aspartic acid in catalysis.

Steady-State Kinetic Characterization of the Mutant Enzymes. To further probe the function of Asp128, three mutant Stp1, D128A, D128E and D128N, were prepared using site-directed mutagenesis. The mutant enzymes were expressed in *E. coli* and purified to homogeneity as described previously for the wild type Stp1 (Zhang et al., 1995). All of the mutant enzymes had chromatographic properties identical to those of the wild type enzyme. Since Asp128 is located on a surface loop, it is unlikely that conservative substitutions at this residue would produce significant perturbations in the overall protein structure. Indeed, comparison of the ^{1}H NMR spectrum of the D129A mutant protein with that of the wild type bovine enzyme indicated minimal structural perturbations by the mutation (Taddei et

Table 1: Comparison of Steady-State Kinetic Parameters of Wild Type and Mutant Enzymes at pH 6.0 and 30 °C[a]

enzymes	k_{cat} (s^{-1})	K_m (mM)	K_i[b] (mM)
WT	3.65 ± 0.26	0.084 ± 0.006	2.4 ± 0.3
D128E	0.54 ± 0.02	0.097 ± 0.014	2.5 ± 0.4
D128A	0.0056 ± 0.0002	0.032 ± 0.004	4.8 ± 0.6
D128N	0.0090 ± 0.0003	0.19 ± 0.015	10.4 ± 1.5

[a] All measurements were made using *p*NPP as a substrate. Buffer used was pH 6.0, 50 mM succinate, 1 mM EDTA, and the ionic strength was kept at 0.15 M, adjusted by NaCl. [b] Competitive inhibition constants for inorganic phosphate.

al., 1994; Zhang, Z., et al., 1994). Inorganic phosphate, a product of the enzyme catalyzed reaction, is a competitive inhibitor of the enzyme (Zhang & Van Etten, 1991a). In the structure of the bovine enzyme, the phosphate ion forms an extensive hydrogen-bonding network to the main chain amide groups of the phosphate binding loop as well as to the side chain of Arg18 in the active site (Zhang, M., et al., 1994). We compared the binding affinity of the wild type Stp1 as well as the mutants for inorganic phosphate (Table 1). Stp1 has a K_i value of 2.4 mM for phosphate at pH 6. The affinity of D128E for phosphate is similar to that of the wild type enzyme, suggesting that a Glu residue can substitute for an Asp residue at position 128 with retention of effective phosphate binding. The K_i value for the D128A and D128N is increased 2- and 4.3-fold respectively. An increase of 3-fold in K_i value was also observed for the D129A mutant of the bovine enzyme (Taddei et al., 1994; Zhang, Z., et al., 1994). This may be consistent with the loss of a hydrogen bond between the phosphate and the carboxyl group of Asp128.

The effects of substitutions at Asp128 on steady-state kinetic parameters using *p*NPP as a substrate at pH 6 and 30 °C are summarized in Table 1. For the three Stp1 mutants only moderate alterations in K_m were noticed. In contrast, more dramatic reductions in k_{cat} value were observed. Thus D128A and D128N displayed a k_{cat} that was 650- and 400-fold lower than that of the wild type enzyme, respectively. A decrease in k_{cat} of more than 2000-fold was detected for the D129A mutant of the bovine enzyme at 37 °C and pH 5−5.5 (Taddei et al., 1994; Zhang, Z., et al., 1994). This is consistent with the proposal that Asp128 serves a critical role in Stp1 catalysis. Interestingly, the k_{cat} value for D128E was only 6.7-fold decreased in comparison with the wild type enzyme. Replacement of Asp with Glu or vice versa generally leads to a reduction of up to 3 orders of magnitude in catalytic efficiency in enzymes that require a carboxylate group as a general acid/base (Straus et al., 1985; Hibler et al., 1987; Zawrotny & Pollack, 1994; Leung et al., 1994). The fact that D128E retained 15% of the native catalytic activity indicates that the active site of Stp1 is fairly flexible and can tolerate a one methylene unit lengthened side chain as long as the carboxylate functionality is preserved. If Asp128 is involved in both the phosphoenzyme formation and its breakdown, then modification of this acid/base residue may affect the rates of both steps. The fact that different effects are observed for different mutations suggests that the extent to which each step is affected is not necessarily equivalent. Further mechanistic insights from these mutants can be obtained by studying the effects of the mutations on the individual steps of the reaction using pre-steady-state techniques.

Pre-Steady-State Kinetics. Pre-steady-state stopped-flow kinetic analyses of Stp1 and its Asp128 mutant-catalyzed hydrolysis of *p*NPP were conducted at pH 6.0 and 30 °C. The complete time courses of the reaction as monitored at 410 nm for the production of *p*-nitrophenolate are shown in Figure 1. The individual rate constants for the formation (k_2) and breakdown (k_3) of the intermediate (E−P in Scheme 1) were calculated as described in Materials and Methods. Table 2 summarizes all of the pre-steady-state kinetic constants for the wild type Stp1 as well as its Asp128 mutants. The k_{cat} values (*A* in Table 2) determined from the burst kinetic experiments are nearly identical to those measured under steady-state conditions (Table 1). There is good agreement between the directly observed amplitude of the burst with the theoretically predicted value under the experimental conditions. We noticed that the rate of the burst formation (*b*) for the wild type enzyme observed in this study is slightly slower than that from a previous measurement (Zhang et al., 1995), which may be due to variations in the experimental conditions. The rate for the intermediate formation (k_2) is 30-fold faster than the rate of the intermediate decomposition (k_3) for the wild type enzyme. For D128E, k_2 is 11-fold faster than k_3. Overall, these results establish that the rate-limiting step of Stp1 and its D128E mutant-catalyzed hydrolysis of *p*NPP is k_3, the decomposition of the phosphoenzyme intermediate.

Burst kinetics are also observed with the D128A mutant. Interestingly, in this case k_2 is only 1.5-fold faster than k_3, indicating that both k_2 and k_3 contribute to k_{cat}. This is apparent both from the much less pronounced burst (Figure 1C) and the low ratio between the observed burst size and the enzyme concentration (Table 2). Thus, our rapid kinetic results on the D128A mutant do not support either of the two previous steady-state kinetic studies of the D129A mutant of the bovine enzyme. If the rate of the overall reaction were controlled by k_2 (Zhang, Z., et al., 1994), then no burst ought to be observed. On the other hand, if k_2 were 24.5-fold faster than k_3 (Taddei et al., 1994), then a nearly stoichiometric burst should be observed. We did not observe a burst in the D128N-catalyzed hydrolysis of *p*NPP (Figure 1D), suggesting that the intermediate forms much more slowly than it breaks down. It appears that the rate-limiting step changes from k_3 to k_2 when Asp128 is changed to an Asn. These conclusions are further supported by results from partition experiments and leaving group dependence experiments (discussed below).

Our rapid kinetic analyses of the wild type Stp1 and its Asp128 mutants show that Asp128 is involved in both the formation and the breakdown of the phosphoenzyme intermediate. This is consistent with the differential effects on the individual steps due to substitutions at Asp128. Thus, the rate of intermediate formation drops 17-, 7480-, and 11900-fold when the general acid Asp128 is replaced with a Glu, Ala, or Asn, respectively. The rate of phosphoenzyme intermediate decomposition is decreased 6.2- and 380-fold, respectively, for the D128E and D128A mutants. k_3 cannot be determined directly for the D128N-catalyzed hydrolysis of *p*NPP since no detectable burst is observed. However, a reasonable lower limit for k_3 can be estimated. If we assume that the detection limit of the stopped-flow spectrophotometer is 0.001 absorbance units, then the minimal burst that can be observed under the experimental conditions would be 0.75 μM, which suggests that k_3 is at least 10-fold faster than k_2

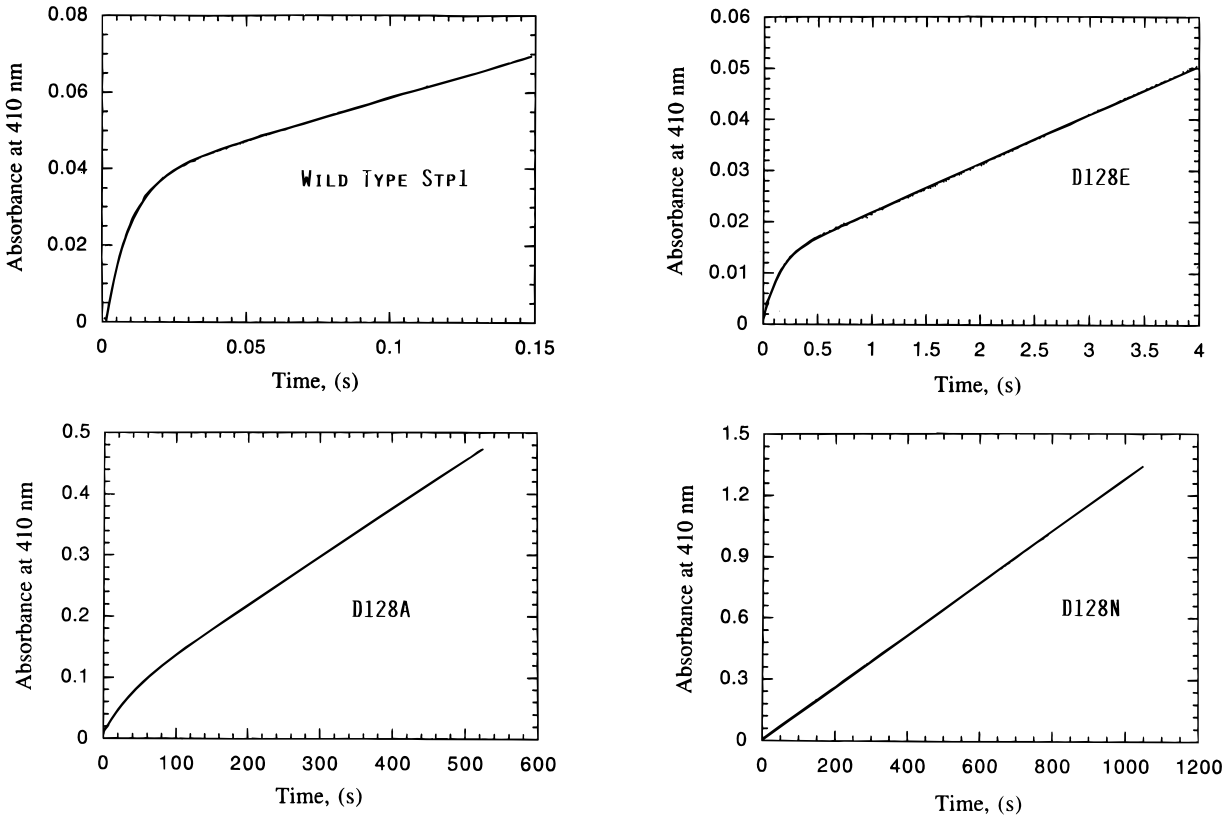


FIGURE 1: Burst kinetics observed with yeast Stp1 and *p*NPP at pH 6 and 30 °C. The *p*NPP concentration was 5 mM. Each stopped-flow trace was an average of at least six individual experiments. The solid line represents a theoretical fit of the data to the equation [*p*-nitrophenolate] $= At + B(1 - e^{-bt}) + C$. A, wild type Stp1, concentration 43.6 μM; B, D128E, concentration 12.3 μM; C, D128A, concentration 93.6 μM; and D, D128N, concentration 95.7 μM.

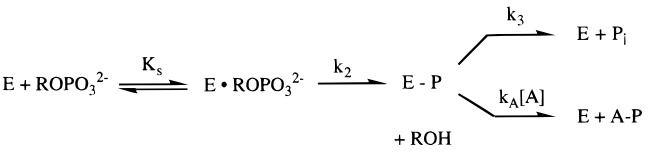
Table 2: Summary of Pre-Steady-State Kinetic Parameters of Wild Type and Mutant Enzymes at pH 6.0 and 30 °C[a]

enzymes	A (s^{-1})	b (s^{-1})	B (μM)	$B/[E_o]$	k_2 (s^{-1})	k_3 (s^{-1})
WT	3.85	123	37.9 (39.4)[b]	0.87 (0.90)[c]	119	4.0
D128E	0.582	7.56	9.20 (9.96)[b]	0.75 (0.81)[c]	6.92	0.64
D128A	0.00633	0.0264	34.6 (33.7)[b]	0.37 (0.36)[c]	0.0159	0.0105
D128N	0.010		0	0	0.010	0.1[d]

[a] The rate constants for the linear phase (A), the exponential phase (b), and the amplitude of the burst (B) are obtained from the direct fitting of the experimental data to the equation [*p*-nitrophenolate] $= At + B(1 - e^{-bt})$. The individual rate constants for the enzyme phosphorylation (k_2) and dephosphorylation (k_3) can be determined from $b = k_2 + k_3$ and $A = k_2k_3/(k_2 + k_3)$, respectively. [b] The theoretical sizes of the burst are listed in parentheses and are calculated from $B = E_o[k_2/(k_2 + k_3)]^2/(1 + K_m/S_o)^2$. [c] The theoretical ratio between the amplitude of the burst and the active site concentration of the enzyme under the experimental conditions. [b] k_3 value for D128N was a lower limit of the estimated value (see text).

as calculated from the equation $B = E_o[k_2/(k_2 + k_3)]^2/(1 + K_m/S_o)^2$. Thus, k_3 for D128N is at most reduced 40-fold compared with the wild type enzyme. It is interesting to note that, in general, substitutions at position 128 have a more profound effect on the step leading to the intermediate formation, as compared to its decomposition. The differential effects on k_2 and k_3 caused by the D128A and D128N mutants are also interesting. It is likely that in the D128A mutant solvent water molecules may fill the space of the missing carboxyl group. Neither a water nor a carboxyamide group is a good proton donor for effective assistance of the leaving group departure. Since the side chain of an Asn is isosteric to that of an Asp and can still form hydrogen bonds, the D128N mutant may be a much more effective catalyst than D128A in activating or positioning the water molecule for nucleophilic attack of the phosphoenzyme intermediate. Furthermore, the Asn side chain could be generally better at keeping the overall active site architecture intact.

Scheme 2



Partition Experiment. The selective influence of a nucleophilic acceptor on the kinetics of the Stp1 catalyzed hydrolysis of *p*NPP was examined. The ability to catalyze phosphate transfer and partition reactions is a common feature for phosphate ester hydrolases that involve a phosphoenzyme intermediate (Fersht, 1985). Scheme 2, where E−P is a phosphoenzyme intermediate and A is a phosphoacceptor, demonstrates this approach. If the rate-limiting step is the formation of a covalent intermediate, then the presence of the acceptor will only change the product distribution but not the overall rate. If the rate-limiting step is the hydrolysis of the intermediate, then the presence of the acceptor will increase the rate of the breakdown of the intermediate and hence increase the overall reaction rate. Thus, results obtained from these experiments can provide information of the rate-limiting step for the enzyme-catalyzed hydrolysis reaction. In experiments with Stp1 and *p*NPP conducted in the presence of ethylene glycol, both inorganic phosphate and ethylene glycol phosphate are formed. The overall rate of substrate turnover (hydrolysis plus phosphate transfer) is measured by the production of *p*-nitrophenol whereas the

Table 3: Effect of Ethylene Glycol[a]

enzyme	± 1 M ethylene glycol	k_{cat}[b] (s^{-1})	K_m[b] (mM)	k_{cat}[c] (s^{-1})	K_m[c] (mM)
WT	−	3.65 ± 0.26	0.084 ± 0.006	3.60 ± 0.13	0.081 ± 0.009
	+	13.8 ± 0.6	0.31 ± 0.08	3.58±0.17	0.38 ± 0.07
D128E	−	0.54 ± 0.02	0.097 ± 0.014	0.52 ± 0.05	0.089 ± 0.02
	+	1.67 ± 0.16	0.206 ± 0.15	0.49 ± 0.09	0.185 ± 0.011
D128A	−	0.0056 ± 0.0002	0.032 ± 0.004	0.0055 ± 0.0003	0.031 ± 0.005
	+	0.0071 ± 0.0002	0.040 ± 0.003	0.0051 ± 0.0002	0.039 ± 0.005
D128N	−	0.0090 ± 0.0003	0.19 ± 0.015	0.0090 ± 0.014	0.179 ± 0.012
	+	0.0097 ± 0.0004	0.196 ± 0.020	0.0095 ± 0.004	0.178 ± 0.010

[a] All of the experiments were performed at pH 6 and 30 °C. [b] Determined by the production of *p*-nitrophenol. [c] Determined by the production of inorganic phosphate.

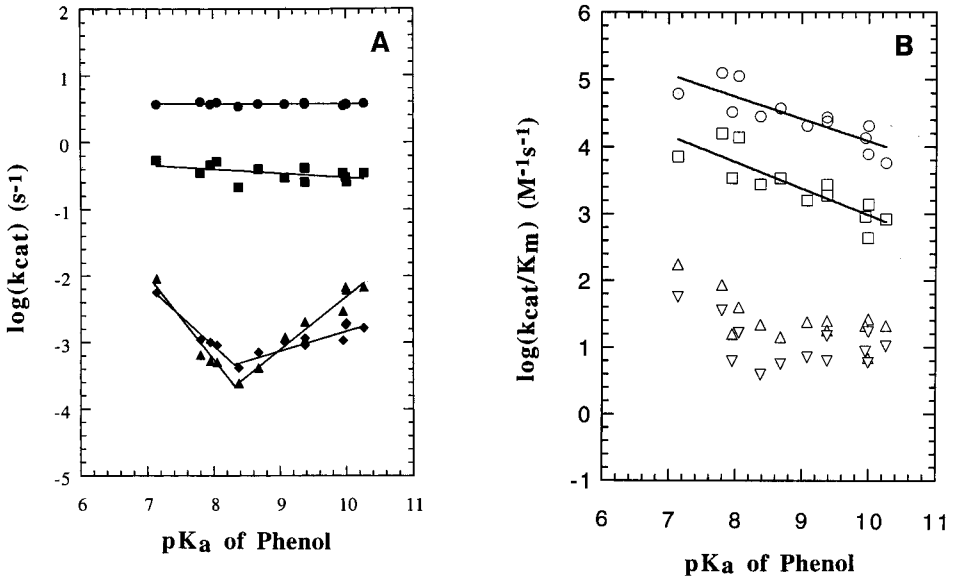


FIGURE 2: Dependence of Stp1-catalyzed hydrolysis of aryl phosphates on leaving group ability. A, k_{cat} vs leaving group pK_a, (●) wild type Stp1, (■) D128E, (▲) D128N, and (◆) D128A. B, k_{cat}/K_m vs leaving group pK_a, (○) wild type Stp1, (□) D128E, (▽) D128N, and (△) D128A. The substrates used were *p*NPP (7.14), 4-methylumbelliferyl phosphate (7.8), 4-acetylphenyl phosphate (8.05), 4-cyanophenyl phosphate (7.95), 3-nitrophenyl phosphate (8.38), 3-chlorophenyl phosphate (9.08), 4-(trifluoromethyl)phenyl phosphate (8.68), β-naphthyl phosphate (9.38), 4-chlorophenyl phosphate (9.38), 4-fluorophenyl phosphate (9.95), phenyl phosphate (9.99), 4-ethylphenyl phosphate (10.0), and 4-methylphenyl phosphate (10.26). Numbers in parentheses refer to the pK_a values of the phenol leaving groups.

hydrolysis rate is measured by the production of inorganic phosphate. As a control, the kinetic parameters of *p*NPP hydrolysis determined by the *p*-nitrophenol assay are similar to those determined by the phosphate assay in the absence of ethylene glycol (Table 3). k_{cat} values for the hydrolysis of *p*NPP as determined by the phosphate assay in the absence of ethylene glycol are also close to those determined in the presence of 1 M ethylene glycol (Table 3). This is because the molarity of water is not changed significantly by the introduction of 1 M ethylene glycol. As shown in Table 3, the overall reaction rate of the wild type Stp1 and the D128E mutant-catalyzed *p*NPP turnover is accelerated 3.8- and 3.1-fold, respectively, by the presence of 1 M ethylene glycol. On the other hand, the overall rate of the D128A-catalyzed *p*NPP turnover is only 1.3-fold faster in the presence of 1 M ethylene glycol, while no appreciable rate acceleration is observed for the D128N mutant. These results indicate that, for the hydrolysis of *p*NPP, k_3 is the rate-limiting step for both the wild type and the D128E mutant and only partially rate-limiting for the D128A mutant. The rate-limiting step for the D128N mutant is probably k_2. These conclusions are consistent with those reached from pre-steady-state experiments described above.

Leaving Group Dependence. We have also examined the effects of leaving group on the wild type as well as the Asp128 mutant-catalyzed reaction. These linear free energy relationships, or Brønsted correlations, provide information about the rate-limiting step and the nature of the transition-state. Figure 2A,B show the Brønsted plots which relate the k_{cat} and k_{cat}/K_m values, respectively, to the pK_a values of the leaving group. For the wild type Stp1 and the D128E mutant, the β_{lg} for k_{cat} [which is the slope of log(k_{cat}) versus pK_a] is -0.003 ± 0.006 and -0.062 ± 0.032, respectively. As shown in Scheme 1, the kinetic parameter $k_{cat} = k_2k_3/(k_2 + k_3)$. The fact that the Stp1-catalyzed hydrolysis of aryl phosphates, substrates that differ markedly in their leaving group pK_a (for example, 7.14 for *p*-nitrophenol and 10.26 for 4-methylphenol), displays identical k_{cat} values is consistent with the rate-determining step being the breakdown of the phosphoenzyme intermediate (k_3). k_3 may also be primarily rate-limiting for D128E since a very small leaving group effect on k_{cat} is observed. These conclusions are in agreement with results from burst kinetic experiments and partition experiments described above.

Nonenzymatic phosphoryl transfer reactions are believed to involve an "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 (Skoog & Jencks, 1984; Bourne & Williams, 1984; Herschlag & Jencks, 1989; Cleland, 1990; Hengge et al., 1994). As a result there is a corresponding negative charge developing on the departing alcoholic or

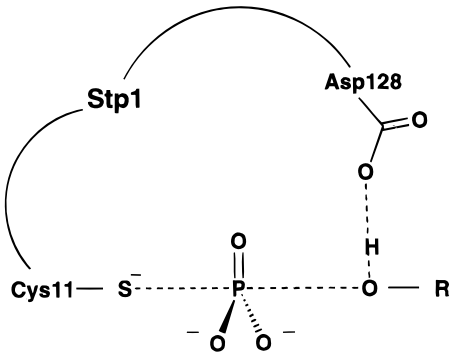


FIGURE 3: Suggested transition-state for the phosphorylation of Stp1.

phenolic oxygen atom that can be stabilized by protonation, a positively charged group, or a metal ion. The low sensitivity to leaving group dependence ($\beta_{lg} = -0.27$) in the phosphate monoester monoanion hydrolysis has been attributed to the protonation of the leaving group (Kirby & Varvoglis, 1967).

Recently, we have measured heavy-atom kinetic isotope effects in the *Yersinia* PTPase and the mammalian PTP1-catalyzed *p*NPP hydrolysis reaction (Hengge et al., 1995). Our results demonstrate that, for both PTPases, (1) P−O bond cleavage is rate-limiting for the k_{cat}/K_m portion of the mechanism; (2) the transition-state for phosphorylation of the PTPases is highly dissociative in character, as is the case for the non-enzymatic reaction; and (3) the proton from the general acid is largely transferred to the bridge oxygen in the transition-state. The primary ^{18}O kinetic isotope effect indicates that P−O bond cleavage is also rate-limiting for k_{cat}/K_m in the Stp1-catalyzed *p*NPP hydrolysis (A. C. Hengge and Z.-Y. Zhang, unpublished result). Like the bovine enzyme (Zhang & Van Etten, 1991b), the kinetic parameter k_{cat}/K_m probably mainly monitors the formation of the phosphoenzyme. The β_{lg} for k_{cat}/K_m [which is the slope of $\log(k_{cat}/K_m)$ versus pK_a] is -0.33 ± 0.06 and -0.40 ± 0.07, respectively, for the wild type and the D128E mutant (Figure 2B). Thus, similar to the specific acid-catalyzed hydrolysis of phosphate monoester, the small value of β_{lg} for reactions of phosphate esters with Stp1 and the D128E mutant is most consistent with protonation of the oxygen atom of the leaving group by the Asp128/Glu128 residue. On the basis of the above discussions, a dissociative transition-state for the phosphorylation of Stp1 is suggested in Figure 3. The P−O bond to the leaving group is largely broken, proton transfer to the leaving group oxygen is correspondingly advanced such that the departing phenol has no charge, and the central phosphoryl group resembles metaphosphate in structure. *The fact that mutations at Asp128 have greater effects on k_2 than k_3 is consistent with a dissociative transition-state: a greater help is needed to facilitate the departure of the leaving group in the phosphoenzyme formation step (k_2) than to activate the nucleophilic water in the phosphoenzyme hydrolysis step (k_3).* The differential effects on k_2 and k_3 by substitutions at Asp128 could also be explained either by the reacting group being held covalently in the correct position for reaction so that little change is expected in k_3 in dephosphorylation or general base catalysis being less necessary for cleavage of the higher energy thiophosphate intermediate.

Nonlinear Brønsted Plots for the D128N- and D128A-Catalyzed Reactions. A biphasic Brønsted relationship with a concave-upward shape was observed for k_{cat} in both the D128N and D128A mutant-catalyzed reactions (Figure 2A). A similar but much less defined biphasic relationship for k_{cat}/K_m was also obtained for the D128N and D128A mutants (Figure 2B). In this case it is difficult to extract a meaningful β_{lg} value. In the structure of the bovine enzyme, Asp129 interacts with the putative bridging ester oxygen in the substrate (Su et al., 1994; Zhang, M., et al., 1994). This interaction may be altered in both the D128N and D128A mutants such that abnormal effects on binding and orientation of the substrate within the active site may obscure a simple dependence of reaction rate on intrinsic reactivity in the term k_{cat}/K_m [for further discussions, see Kirsch (1972) and Hollfelder and Herschlag (1995)].

As shown by rapid kinetics and partition experiments, the rate-limiting step for D128N corresponds to k_2. In the case of D128A, k_2 is only marginally larger than k_3 with *p*NPP as a substrate. When the pK_a of the leaving group is greater than 7.14, k_2 will most likely become rate-limiting for D128A as well. Thus, for both D128N and D128A, consideration of k_{cat} may be more informative, which is primarily dictated by k_2. When the leaving group pK_a is less than 8.3, a strong dependence of k_2 on leaving group pK_a was observed for both D128N and D128A, with a β_{lg} of -1.29 ± 0.17 and -0.90 ± 0.07, respectively (Figure 2A). This indicates that when $pK_a < 8.3$, electron-withdrawing substituents increase the rate of phosphoenzyme intermediate formation. Interestingly, when the leaving group pK_a is greater than 8.3, the β_{lg} for k_2 is $+0.79 \pm 0.07$ and $+0.30 \pm 0.05$, respectively, for the D128N- and D128A-catalyzed reaction. Thus when $pK_a > 8.3$, substrates with electron donating substituents favor the phosphorylation reaction (Figure 2A).

A biphasic linear free energy relationship has generally been interpreted as an indication of change in mechanism or rate-limiting step (Kirsch, 1972; Jencks, 1987; Kempton & Withers, 1992). Curvature in linear free energy relationship is also consistent with a concerted mechanism when there is a change in transition-state structure. According to transition-state theory (Eyring, 1935; Moore & Pearson, 1981), the transition-state is in thermodynamic equilibrium with the reactants. In the transition-state, chemical bonds are in the process of being made and broken. Results from Brønsted analysis of the D128N- and D128A-catalyzed reaction indicate a change in the nature of the transition-state with changing structure of the substrates. The essential feature in the suggested dissociative transition-state of the phosphorylation of Stp1 (Figure 3) is the highly advanced P−O bond fission, coupled with the concerted proton transfer to the leaving group oxygen. In this highly unstable, metaphosphate-like transition-state, a strong electrophilic "pull" on the leaving group would be required for the productive forward reaction. In the wild type Stp1 and the D128E mutant, this is accomplished by the general acid at residue 128. In the Stp1 mutants D128N and D128A, the general acid has been removed. Although in the D128A mutant a water may take the place of a carboxyl group and in the D128N mutant the carboxy amide may retain some hydrogen bonding ability, it is clear that these two mutants cannot stabilize the developing negative charge in the leaving group as efficiently as the wild type and the D128E mutant. This may explain that k_2, instead of k_3, becomes rate-limiting for D128N and D128A. In the trigonal bipyramidal transi-

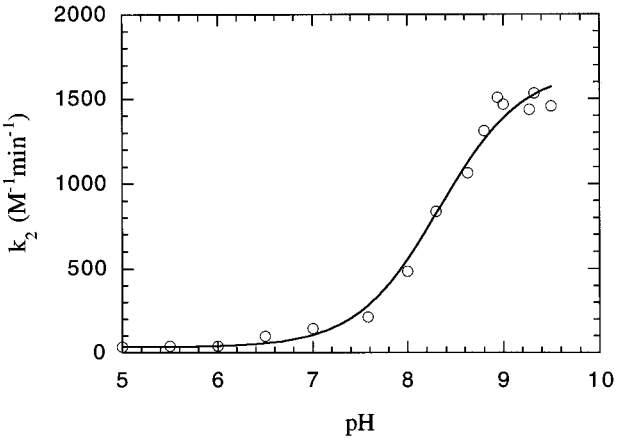


FIGURE 4: pH dependence of the rate of the inactivation of Stp1 by iodoacetate. The experimental conditions were specified under Material and Methods. The line in the figure was obtained by a direct fit of the data to equation $k_2 = k_2^o/(1 + [H^+]/K_a) + k_2'$ where k_2^o is the pH-independence alkylation rate constant for the thiolate anion, k_2' is the pH-independent alkylation rate constant for the sulfhydryl group, and K_a is the acid dissociation constant. This equation was employed for calculation of the pK_a value.

tion-state (Figure 3), the general acid deficient mutant PTPase-catalyzed reaction can either go forward to expel the phenol leaving group or come back to regenerate the active site sulfur nucleophile. In searching for an explanation for the curvature in the Brønsted plots, we determined the pK_a for the active site thiol group. Previously, it was shown that the alkylating agent, iodoacetate, irreversibly inactivated the bovine low M_r PTPase (Camici et al., 1989). The main residue that was labeled by iodoacetate was the active site cysteine. Thus, an apparent pK_a of 8.30 ± 0.06 was determined for the active site thiol[1] from the pH dependency of iodoacetate inactivation of Stp1 (Figure 4), which corresponds exactly to the pK_a value at the break point in the Brønsted plots (Figure 2A).

It is likely that in the D128N- and D128A-catalyzed phosphorylation reaction, the stabilization of the negative charge on the leaving group and the actual P−O bond breaking process are not synchronized. These two processes have different dependencies on the property of the substituents. Substituents that favor protonation/electrostatic interaction (*i.e.*, electron donating) also hinder the expulsion of the leaving group and vice versa. Thus, for substrates with leaving group pK_a values lower than that of the active site thiol (pK_a < 8.3), the P−O bond breaking may be far advanced with little need for charge stabilization and the intrinsic stability of the phenolate anion may be the driving force for reaction. The observed β_{lg} of −0.9 and −1.29 are similar to that obtained for the uncatalyzed hydrolysis of phosphate dianion ($\beta_{lg} = -1.27$; Kirby & Varvoglis, 1967) and are consistent with a highly dissociative transition-state and that the leaving group departs as an anion. *The fact that elimination of the general acid increases the β_{lg} supports Asp128 acting as a proton donor to reduce the effective charge on the bridge oxygen.* When the leaving group pK_a is higher than that of the active site thiol (pK_a > 8.3), the trigonal bipyramidal transition-state, once it is formed, will break down to expel the attacking nucleophile instead of the leaving group and will give back starting material. In this situation, electrophilic assistance is absolutely required for the productive partitioning of the forward reaction. The β_{lg} values of +0.79 and +0.30 for D128N and D128A at pK_a > 8.3 suggest that electrophilic stabilization of the developing negative charge is more important and may precede P−O bond breaking. In other words, the affinity for positive charge and thus the extent of localized charge stabilization provide the driving force for the reaction. One would also predict that the transition-state may become more associative for substrates with leaving group pK_a > 8.3 in the D128N- and D128A-catalyzed reactions. This can be tested by determining the heavy-atom kinetic isotope effects associated with these reactions.

Conclusion. We have demonstrated in this paper that Asp128 is required for both the formation and breakdown of the phosphoenzyme intermediate. Asp128 facilitates the intermediate formation by protonation of the bridge oxygen in the leaving group. To hydrolyze the phosphoenzyme intermediate, Asp128 serves to activate or position the water molecule for nucleophilic attack. The fact that substitutions at position 128 have greater effect on k_2 than k_3 is consistent with a dissociative transition-state for the Stp1-catalyzed reaction. The catalytic functions of Asp128 can to a large extent be restored by a Glu residue. Thus, in both the wild type and the D128E mutant, the presence of an efficient general acid ensures rapid intermediate formation, which is most likely driven by a concerted proton transfer to the bridge oxygen coupled with the P−O bond cleavage in the transition-state. Our data from three independent experiments (burst kinetics, leaving group dependence, and partition experiment) suggest that k_3 is rate-limiting for the wild type and D128E mutant. The removal of the general acid in D128N and D128A has resulted in k_2 being rate-limiting in the overall reaction for most substrates. Leaving group dependence studies of the general acid deficient mutants reinforce the notion that efficient charge stabilization is the driving force for the phosphorylation reaction. The sharp break in the leaving group structure and reactivity correlations of the general acid deficient mutants reveals the opposing effects of substitutions on P−O bond breaking and charge stabilization of the bridge oxygen. Such a nonlinear plot is also diagnostic of a change in the transition-state structure due to alterations in the leaving group structure.

[1] Due to the low catalytic activity of the D128A and D128N mutants, it is difficult to determine a pK_a value for the active thiol in these mutants from the pH dependency of iodoacetate inactivation. However, since Asp128 is located on a surface loop, and since comparison of the ^{1}H NMR spectrum of the D129A mutant protein with that of the wild type bovine enzyme indicated minimal structural perturbations by the mutation, it is unlikely that conservative substitutions at this residue would change significantly the active site thiol pK_a.

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