Roles of Aspartic Acid-181 and Serine-222 in Intermediate Formation and Hydrolysis of the Mammalian Protein-Tyrosine-Phosphatase PTP1[†]

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ABSTRACT: Protein tyrosine phosphatases (PTPases) share a number of conserved amino acid residues, including the active site sequence HCXXGXXRS(T), which are strongly implicated in catalysis. The roles of two conserved active site residues, Asp-181 and Ser-222, were investigated using a combination of site-directed mutagenesis and kinetic analysis in the mammalian PTPase PTP1. The pH profiles for k_{cat}/K_m and k_{cat} of the wild-type enzyme indicate that two ionizable groups, of pK_a values 5.1 and 5.44, must be deprotonated and one group with a pK_a value of 4.93 must be protonated for maximal activity. The group of pK_a value 5.1 is the second ionization of the substrate phosphate moiety. Selective thiolate anion inactivation indicates the residue with pK_a value of 5.44 is C215. The pH-dependent profiles of the D181N mutant during establish the residue with pK_a value of 4.93 to be Asp-181 and suggest that it functions as a general acid phosphoryl transfer to the enzyme. Rapid reaction kinetics of wild-type and D181N mutant enzymes indicate that the formation of the phospho−enzyme intermediate is rate-limiting at pH 7.0 and 30 °C. Enzymes containing the S222A mutation exhibited rapid reaction burst kinetics, strongly suggesting that phospho−enzyme intermediate hydrolysis is fully rate-limiting. The role of the active site S222 is to accelerate the rate of phospho−enzyme intermediate hydrolysis. The kinetic analysis of a third mutant, containing both the D181N and S222A mutations, suggests that D181 also serves as a general base in the breakdown of the phospho−enzyme intermediate.

The level of protein phosphorylation is tightly controlled within the cell. Protein dephosphorylation is accomplished by a family of enzymes known collectively as protein phosphatases. The protein phosphatases fall into distinct structural classes: the serine/threonine specific protein phosphatases that require metals to activate a water molecule during phosphate hydrolysis and protein tyrosine phosphatases (PTPases)[1] that require no metals but use a nucleophilic cysteine in the cleavage of the monophosphate−ester bond (Lohse *et al.*, 1996; Denu *et al.*, 1996b).

Human PTP1B was the first PTPase to be purified (Charbonneau *et al.*, 1988; Tonks *et al.*, 1988a,b), and the three-dimensional structure of the 322 amino acid catalytic domain has been solved (Barford *et al.*, 1994). The rat structural homologue, PTP1, had been cloned (Guan *et al.*, 1990) and characterized (Zhang, 1995). The sequences between amino acids 1 and 322 of PTP1 and PTP1B are 97% identical (Guan *et al.*, 1990).

Considerable effort has been devoted to understanding the structure, function, and biological properties of the PTPases. Using site-directed mutagenesis in conjunction with a detailed kinetic analysis, it has been previously shown in the dual-specificity PTPase VHR (Denu *et al.*, 1995) and the *Yersinia* PTPase (Zhang *et al.*, 1994a,c) that a conserved aspartic acid residue serves as a general acid in the catalytic mechanism. The function of a conserved serine residue, present within the active site of VHR, was found to be involved in accelerating intermediate hydrolysis (Denu & Dixon, 1995).

In the current study we demonstrate that Asp-181 functions as a general acid during formation of phospho−enzyme intermediate and a S222A mutation dramatically slows the breakdown of this intermediate. This has allowed us to produce a double mutant containing substitutions S222A and D181N to establish that Asp-181 may also serve as a general base, abstracting a proton from water in the breakdown of the phospho−enzyme intermediate. These general features of the catalytic mechanism appear to be used by most all PTPases.

MATERIALS AND METHODS

Vector Construction and Site-Directed Mutagenesis. The expression vector for PTP1 (residues 1−323) was constructed by ligating an Eco*RI*/Sac*I* fragment from pKG-PTP323 (Guan & Dixon, 1991) into pUC118. A Xho*I*/Bam*HI* fragment from the pUC118-PTP1 plasmid was ligated with a Nde*I*/Xho*I* fragment from the cDNA encoding a partial sequence of PTP1 into a pT7-7 vector previously digested with Nde*I*/Bam*HI*. The sequence of the recombinant expression plasmid encoding PTP1 amino acids (1−323) was verified by DNA sequencing.

Site-directed mutagenesis was carried out using the BioRad Muta-gene method. The following oligonucleotides were

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[1] Abbreviations: PTPase, protein-tyrosine-phosphatase; PTP1, protein-tyrosine-phosphatase 1; VHR, vaccinia H1-related; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane; *p*NPP, *p*-nitrophenylphosphate; D, aspartic acid; C, cysteine; S, serine; A, alanine; R, arginine.

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synthesized in an Applied Biosystems model 391 DNA synthesizer and used to construct the S222A, D181N, and S222A/D181N mutant enzymes. The underlined base indicates the change from the naturally occurring nucleotides: S222A, 5′-ATTGGCAGGGCAGGGACCTTC-3′; and D181N, ACCACCTGGCCTAACTTTGGA. The changes were verified by DNA sequencing.

Overexpression and Purification. The pT7-7-PTP1/WT, pT7-7-PTP1/D181N, pT7-7-PTP1/S222A, and pT7-7-PTP1/D181N/S222A plasmids were used to transform competent *Escherichia coli* strain BL21/DE3. The transformed bacteria were grown on 2×YT plates containing 100 μg/mL ampicillin. Overnight 10 mL cultures originating from isolated colonies were used to inoculate 1 L of 2×YT containing 140 μg/mL ampicillin. When the growth reached an optical density of 0.8 at 600 nm, isopropyl β-D-thiogalactopyranoside was added at 100 μg/mL and the bacteria were grown for an additional 6 h. The cells were harvested by centrifugation at 5000*g* for 15 min, resuspended in a 15 mL/L culture of 25 mM tris(hydroxymethyl)aminomethane (Tris), 2 mM (ethylenedinitrilo)tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), pH 7.4 and lyzed by pressure (1200 psi) using a 40 mL French press cell (American Instrument Co. Inc.). The cell debris was removed by centrifugation at 27000*g* for 20 min, and the supernatant was decanted away from the pellet. Ammonium sulfate was added to the supernatant (35 mL) to a concentration of 1.7 M, stirred for 15 min at 4 °C, and centrifuged for 20 min at 27000*g*. The supernatant was decanted away from the pellet and loaded onto a 50 mL phenyl-Sepharose (Sigma) column previously equilibrated with 25 mM Tris, 1.7 M ammonium sulfate, 2 mM EDTA, 1 mM DTT, pH 7.4, at 4 °C. After extensive washing with the same buffer (500 mL), the protein was eluted using an ammonium sulfate gradient from 1.7 to 0 M in the same Tris buffer. The wild-type, D181N, S222A, and D181N/S222A mutant enzymes eluted around 400 mM ammonium sulfate. Fractions containing phosphatase activity toward *p*-nitrophenylphosphate (*p*NPP) were pooled and dialyzed against a 40× vol of 25 mM Tris, 2 mM EDTA, 1 mM DTT, pH 7.4, at 4 °C. The resulting solution was then loaded onto a S20 Bio-Rad biologic column previously equilibrated with 25 mM Tris, 2 mM EDTA, 1 mM DTT, pH 7.4, at 4 °C, extensively washed (100 mL) with the same buffer, and eluted using a linear gradient from 0 to 0.6 M sodium chloride. All enzymes eluted at 250 mM sodium chloride. Fractions exhibiting maximal phosphatase activity toward *p*NPP were analyzed by 10% sodium doceyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). All fractions which contained only a single species corresponding to wild-type protein, D181N-PTP1, S222A-PTP1, and D181N/S222A-PTP1 were pooled. The enzymes were stored at 4 °C in 25 mM Tris, 2 mM EDTA, 1 mM DTT containing 300 mM sodium chloride, pH 7.4.

Protein-Tyrosine-Phosphatase Assays. All activity assays were performed using the artificial substrate *p*NPP. A component buffer consisting of 50 mM Tris, 50 mM Bis-Tris, and 100 mM acetate was used for all kinetic assays since it has a constant ionic strength over a pH range of 4.5−9.0 (Ellis & Morrison, 1982). The phosphatase reaction was followed by measuring the increase in absorbance at 405 nm due to the production of the *p*-nitrophenol (Zhang & Van Etten, 1991). Initial rates were determined from the change in absorbance upon addition of 1 M sodium hydroxide. Rates were determined over the linear region using a molar extinction coefficient of 18 000 M^{-1} cm^{-1} for the *p*-nitrophenylate anion. Initial rates at various initial substrate concentrations were then fit directly to the Michaelis−Menton equation using the nonlinear least-squares program Kinetasyst for the Macintosh (IntelliKinetics, State College, PA). Fitting of the pH-dependent data to eqs 2−4 was accomplished using NonLin, a nonlinear least-squares fitting program for the Macintosh (R. Brenstein, Southern Illinois University) derived from a computer code developed by Johnson & Frasier (Johnson and Frasier, 1985). In eqs 2−4, C is the pH-independent value of either k_{cat} or k_{cat}/K_m, H is the proton concentration, and K_a, K_b, and K_c are the ionization constants of the groups involved in the reaction:

$$v = k_{cat}S/(S + K_m) \quad (1)$$

$$v = C/(1 + H/K_a) \quad (2)$$

$$v = C/[(1 + H/K_a)(1 + K_b/H)] \quad (3)$$

$$v = C/[(1 + H/K_a)(1 + K_b/H)(1 + H/K_c)] \quad (4)$$

Circular Dichroism of Wild-Type and Mutant PTPases. Circular dichroic analysis was perfomed on the wild-type and mutant proteins using a Jasco-710 spectropolarimeter at a concentration of 0.1 mg/mL (2.67 μM) in 5 mM phosphate buffer, pH 7.0.

Inhibition by Phosphate. The inhibition constant, K_i, for phosphate was determined for the wild-type and S222A mutant enzymes in the following manner. At various fixed concentrations of inhibitor, the initial velocity at different *p*NPP concentrations was measured as described above. The inhibition of the wild-type enzyme was competitive with respect to substrate, and the data were fit using Kinetasyst (eq 5) to yield the inhibition constant:

$$v = V_{max}S/[K_m(1 + I/K_i) + S] \quad (5)$$

Enzyme Inactivation by Iodoacetate. The apparent second-order rate constant for inactivation of the wild-type enzyme by iodoacetate was determined using the following method. Five different iodoacetic acid concentrations ranging from 0.1 μM to 1 mM were used within a pH range of 4.5−7.4. The inactivation reactions were initiated by adding 10 μL of a 43 μM solution of wild-type PTP1 to a 290 μL solution of varied iodoacetic acid concentrations at a specified pH. Aliquots were taken at various time points between 15 s and 1 min and immediately added to 300 μL of a 100 mM acetate buffer, pH 5.4, containing 25 mM *p*NPP to assay the remaining enzyme activity. Each reaction was quenched by the addition of 700 μL of 1 M sodium hydroxide, and the absorbance at 405 nm was measured. The fraction of remaining activity was determined using a control reaction which contained no iodoacetic acid. The log value of the remaining activity was plotted at each time point, and the slope of the least-squares fit was determined at each iodoacetic acid concentration. The slopes were then plotted as a function of iodoacetic acid concentration and the data fit to a line, the inactivation rate being the slope of this line. This analysis was repeated at each pH. The rates were plotted as a function of pH and fitted using eq 2 to obtain the maximal rate of inactivation and the apparent pK_a value of the catalytic thiol.

Table 1: Purification of PTP1

purification step	sample vol (mL)	total protein (mg)	total activity	specific activity	percent activity
French press	31	2080	12200	5.87	100
ammonium sulfate	32	1420	10500	7.39	86
phenyl-Sepharose	90	257	4680	18.2	38
Bio-Scale S20	24	21.1	3621	172	30

Rapid Reaction Kinetics. The wild-type, D181N, S222A, and D181N/S222A mutant enzymes were rapidly mixed with *p*NPP in a High Tech stopped-flow spectrophotometer at pH 7, 30 °C. The concentration of *p*NPP was at least 5-fold higher than the K_m value. The concentrations of D181N, S222A, and D181N/S222A mutant enzymes were approximately 23, 10, and 5 μM. The enzyme concentrations used for the wild-type enzyme were 11, 8.6, and 2.6 μM. Enzyme concentrations were determined by using an E_{280nm} of 4.64 mM^{-1} cm^{-1}. Product formation (*p*-nitrophenol) was monitored at 405 nm. The data were fit to eq 6 using the nonlinear least-squares fitting capability of the kinetics software KISS: *A* is the amplitude of the burst, *k* is the first-order rate of the burst, *B* is the slope of the linear portion of the curve, *C* is the intercept of the linear portion of the curve, and *t* is time calculated in seconds.

$$\text{absorbance} = Ae^{(-kt)} + Bt + C \quad (6)$$

The amplitude (in absorbance units) was converted to concentration of *p*-nitrophenylate formation at pH 7 using the Henderson−Hasselbach equation, a pK_a value of 7.1, and an E_{405nm} of 8.9 mM^{-1} cm^{-1} for *p*-nitrophenol. The correlation between the concentration of *p*-nitrophenol "burst" and final enzyme concentration was determined by linear least-squares fitting.

Dissociation Constant of S222A and D181N/S222A PTP1. Using the stopped-flow spectrophotometer, the dissociation constant (K_d) for the S222A and D181N/S222A mutant proteins was determined by varying substrate concentration at pH 7.0 and 30 °C. The burst rate (*k* in eq 6) was plotted as a function of substrate concentration and fitted to eq 7, where k_3 is the first-order rate constant for intermediate formation and K_d is the dissociation constant:

$$k(\text{burst rate}) = k_3[S]/(K_d + [S]) \quad (7)$$

RESULTS

Purification of Wild-Type, D181N, S222A, and D181N/S222A PTP1. The wild-type enzyme, D181N, S222A, and D181N/S222A proteins were purified using an identical purification scheme (Table 1). All of the enzymes were purified to apparent homogeneity using ammonium sulfate precipitation and by hydrophobic and cation exchange chromatography (Figure 1). The recombinant wild-type PTP1 was purified 30-fold (Table 1), for an overall yield of 30%. Recoveries ranged from 15 to 25 mg of protein from 3 L of culture broth.

Characterization of the Wild-Type and Mutant PTP1 Proteins. The circular dichroic spectra of wild-type, D181N, S222A, and D181N/S222A enzymes were determined to investigate whether the mutations altered the structure of the expressed proteins. Figure 2 shows that the far-UV spectra have no secondary structural variations, and each protein displayed a similar spectrum.

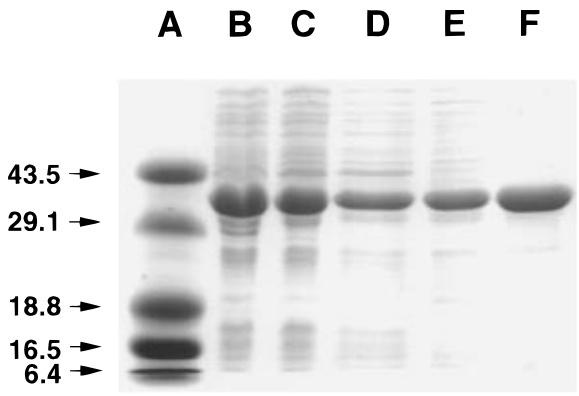


FIGURE 1: Purification for wild-type PTP1 as assessed by SDS−PAGE: lane A, molecular weight standards with molecular weights designated to the left expressed in kDa; lane B, whole cell homogenate; lane C, soluble fraction after centrifugation at 27 000g; lane D, soluble fraction after ammonium sulfate precipitation at 1.7 M; lane E, active fraction from phenyl-Sepharose chromatography; lane F, purified wild-type PTP1 from S20 cation exchange chromatography.

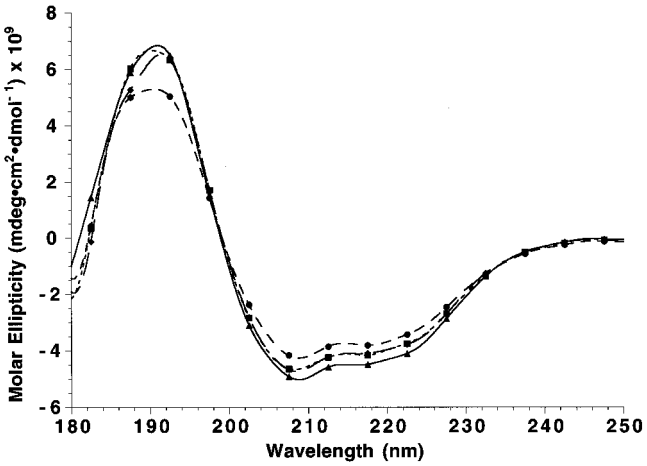


FIGURE 2: Circular dichroic spectra of wild-type and mutant phosphatases. Circular dichroic analysis was done on the wild-type (●), S222A (■), D181N (▲), and D181N/S222A (◆) at a concentration of 0.1 mg/mL (2.67 μM) in 5 mM phosphate buffer, pH 7.0, at 25 °C.

To further verify that these mutations did not alter structure, the Michaelis constant (K_m) for *p*NPP was determined for each protein and the inhibition constant (K_i) for phosphate was determined for the wild-type and S222A mutant enzymes. Fitting to eq 1 generated K_m values for the wild-type, D181N, S222A, and D181N/S222A enzymes, at pH 7.0, of 0.291, 0.504, 6.25 and 3.71 mM, respectively. The inhibition was competitive for both the wild-type and S222A enzymes. Fitting to eq 5 yielded a K_i of 2.77 mM for wild-type and 1.43 mM for S222A enzymes.

Identification of Critical Ionizations for PTP1-Catalyzed Reaction. All PTPases contain conserved amino acid residues whose ionizations are critical in catalysis. To establish the existence of such functional groups, the kinetic parameters k_{cat} and k_{cat}/K_m were determined as a function of pH using *p*NPP as substrate. The k_{cat}/K_m parameter is the apparent second-order rate constant that monitors the reaction beginning with binding of free substrate to free enzyme and all events up to and including the first irreversible step. Ionizations that are reflected in this pH profile are therefore

Table 2: Summary of Kinetic Parameters for Wild-Type and Mutant PTP1 Proteins

enzyme	parameter	eq	value, C	pK_1	pK_2	pK_3
wild-type	$k_{cat/Km\ (M^{-1}\ s^{-1})}$	4	1.40×10^6	5.1	5.44 ± 0.07	4.93 ± 0.10
S222A	$k_{cat/Km\ (M^{-1}\ s^{-1})}$	4	2.64×10^4	5.1	6.36 ± 0.06	4.59 ± 0.08
D181N	$k_{cat/Km\ (M^{-1}\ s^{-1})}$	4	821	5.1	5.31 ± 0.14	8.60 ± 0.12
D181N/S222A	$k_{cat/Km\ (M^{-1}\ s^{-1})}$	4	455	5.1	6.65 ± 0.20	8.29 ± 0.18
wild-type	$k_{cat\ (s^{-1})}$	3	108		4.73 ± 0.06	6.75 ± 0.07
S222A	$k_{cat\ (s^{-1})}$	3	0.426		4.82 ± 0.11	8.27 ± 0.13
D181N	$k_{cat\ (s^{-1})}$	2	0.477		4.77 ± 0.21	
D181N/S222A	$k_{cat\ (s^{-1})}$	2	2.52×10^{-2}		5.47 ± 0.12	
wild-type	$k_{inact\ (M^{-1}\ s^{-1})}$[a]	2	7.13		5.57 ± 0.12	
wild-type	$K_{i\ (mM)}$	6	2.77			
S222A	$K_{i\ (mM)}$	6	1.43			

[a] k_{inact} is the apparent second-order rate constant for the inactivation by iodoacetic acid.

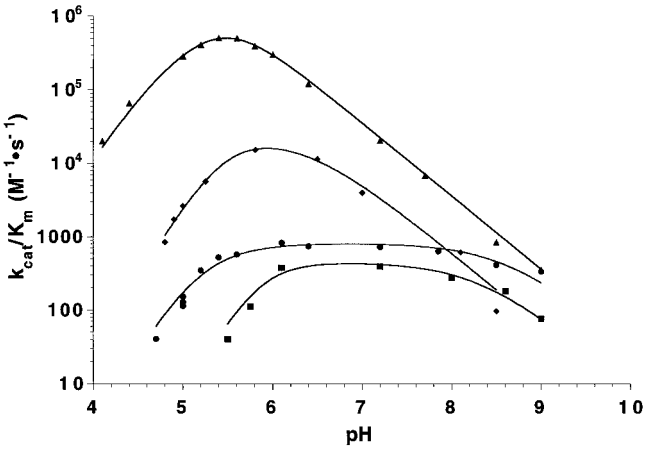


FIGURE 3: Effect of pH on the k_{cat}/K_m value of wild-type and mutant phosphatases: wild-type PTP1 (▲), S222A (◆), D181N (●), and S222A/D181N (■). The buffer consisted of 0.1 M acetate, 0.05 M Tris, and 0.05 M Bis-Tris, and the reactions were run at 30 °C.

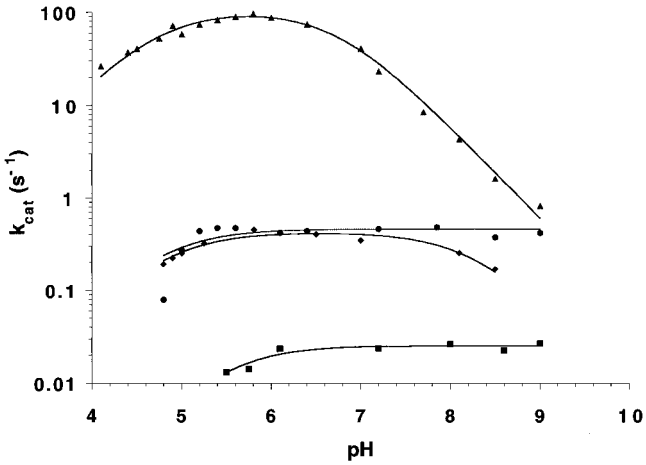


FIGURE 4: Effect of pH on the k_{cat} value of wild-type and mutant PTP1: wild-type PTP1 (▲), S222A (◆), D181N (●), and S222A/D181N (■). The buffer consisted of 0.1 M acetate, 0.05 M Tris, and 0.05 M Bis-Tris, and the reactions were run at 30 °C.

important for binding and/or catalysis. The pH dependency of k_{cat} reflects amino acid ionizations associated with the rate-determining step(s) in the overall reaction.

The k_{cat}/K_m pH profiles for the wild-type and mutant proteins are shown in Figure 3. All of the plots ascend on the acidic side with a slope of 2 and decrease on the basic side with a slope of −1 indicating each enzyme has two groups that must be deprotonated and one that must be protonated for maximal activity. To determine the pK_a values of these groups, each data set was fitted to eq 4. It has been previously shown that a pK_a value of 5.1 corresponds to the second ionization of the phosphate moeity of *p*NPP (Zhang *et al.*, 1994a), and thus it was fixed in the final analyses. The results of these analyses are listed in Table 2. Because the enzyme denaturates at low pH, the importance of additional groups with pK_a values < 4.5 cannot be ruled out.

The wild-type enzyme has two groups with pK_a values of 5.1 and 5.44 that must be deprotonated and a group with a pK_a value of 4.93 that must be protonated for activity. The pH-independent value of k_{cat}/K_m was 1.40×10^6 M^{-1} s^{-1}, and pH optimum was 5.5. The pH dependency of the k_{cat}/K_m for the S222A mutant is very similar to that of the wild-type enzyme with the pH-independent value of 2.64×10^4 M^{-1} s^{-1} and a pH optimum of 5.9 (Figure 3). Two groups with pK_a values of 5.1 and 6.36 must be deprotonated and a group with a pK_a value of 4.59 must be protonated for activity. The pH-independent value for the k_{cat}/K_m of the D181N was 821 M^{-1} s^{-1} and exhibited a broad pH optimum between pH values of 5.31 and 8.60 (Figure 3). Two ionizable groups of the D181N protein, with pK_a values of 5.1 and 5.31, must be deprotonated and an ionizable group with a pK_a value of 8.6 must be protonated for maximal activity. The pH profile of k_{cat}/K_m for the D181N/S222A mutant establishes that two ionizable groups, with pK_a values of 5.1 and 6.65, must be deprotonated and one residue requiring protonation with a pK_a value of 8.29 are required for optimal activity. The pH-independent k_{cat}/K_m value of the D181N/S222A protein was 455 M^{-1} s^{-1}.

Similarly the k_{cat} values were determined as a function of pH for the wild-type, S222A, D181N, and S222A/D181N enzymes (Figure 4). Since k_{cat} describes the reaction of the enzyme−substrate complex, these pK_a values are apparent values and need not agree with values associated with the free enzyme. The data for wild-type enzyme indicated that two ionizable groups are required for catalysis. The group that must be deprotonated has a pK_a value of 4.73, and the other, with a pK_a value of 6.75, must be protonated. The pH-independent k_{cat} value of the wild-type enzyme was 108 s^{-1}. The S222A mutant had one ionizable group requiring deprotonation for maximal activity (pK_a value of 4.82), and another group requiring protonation had a pK_a value of 8.27. The pH-independent value for k_{cat} was 0.426 s^{-1}. The k_{cat} rate profile of the D181N mutant showed no pH dependence above pH 5.5. The pH-independent value was 0.477 s^{-1}. The single pK_a value of 4.77 for a group on the D181N mutant protein that must be deprotonated agrees well with the values of 4.73 and 4.82 obtained for the wild-type and S222A enzymes, respectively (Table 2). The k_{cat} pH rate

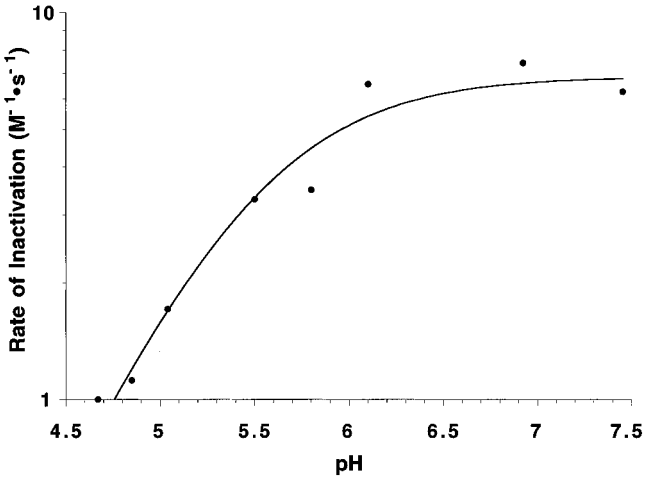
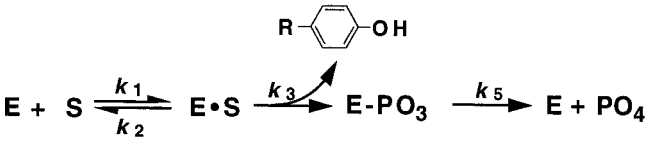


FIGURE 5: pH dependency of the rate of inactivation of wild-type PTP1 by iodoacetate. The data were fit to eq 2; the results are detailed in Materials and Methods and given in Table 1.

Scheme 1: Kinetic Mechanism



profile for the S222A/D181N enzyme also displayed pH independence above 6.1. The pH-independent value was 0.0252 s^{-1}.

pK_a Value Determination of the Active Site Thiolate Anion. Iodoacetic acid has been shown to selectively carboxymethylate the active site nucleophilic cysteine of many PTPases and can be used to determine its apparent pK_a value (Pot & Dixon, 1992; Zhang & Dixon, 1993; Denu *et al.*, 1995). To investigate if the enzymatic group observed in the pH rate profiles with a pK_a value of 5.44 was the active site thiolate (C215), the apparent second-order rate constant for iodoacetic acid inactivation was plotted as a function of pH (Figure 5). The data were fitted to eq 2 yielding a pK_a value of 5.57 which is in good agreement with the pK_a values of 5.44 and 5.31 observed from the wild-type and D181N mutant k_{cat}/K_m profiles, respectively (Table 2).

Rapid Reaction Kinetics of Wild-Type, S222A, D181N, and D181N/S222A PTP1. PTPases are proposed to proceed through a two-step reaction mechanism (Scheme 1) *via* the formation (k_3) and subsequent breakdown (k_5) of a thiol−phosphate−enzyme intermediate (Zhou *et al.*, 1994; Denu & Dixon, 1995). If the rate of intermediate formation is considerably faster than the rate of its breakdown, then a "burst" of product formation could be monitored when enzyme and substrate are rapidly mixed. To explore this possibility, the wild-type, D181N, S222A, and D181N/S222A enzymes were analyzed using a stopped-flow spectrophotometer and the formation of the product, *p*-nitrophenol, was monitored at 405 nm, pH 7, and 30 °C. As seen in Figure 6A,B, no significant "burst" was detected with the wild-type and D181N mutant enzymes even at concentrations approaching 25 μM. Substrate concentration was maintained at 25 mM to ensure that the enzymes were saturated with *p*NPP. These data indicate the net rate for formation of the intermediate is equal to or slower than the rate of intermediate hydrolysis for the wild-type and D181N enzymes, respectively. The linear initial velocity values of 20 and 0.27 s^{-1} from this analysis for the wild-type and D181N enzymes were consistent with the k_{cat} values of 13.5 and 0.42 s^{-1} from the steady state analysis at the same pH (Table 3).

The S222A and D181N/S222A mutant enzymes were also subjected to stopped-flow analysis at similar conditions. As is evident in the data presented in Figure 6C,D, a "burst" of *p*-nitrophenol was detected followed by a slow linear rate in each profile. The amplitudes of the bursts were stochiometric with enzyme concentration, and their burst rates (k_3) were 34 and 1.9 s^{-1} for the S222A and D181N/S222A mutants, respectively. The slow linear rates were calculated to be 1.4 and 0.057 s^{-1} for the S222A and S222A/D181N enzymes, respectively. A summary of the rates derived from the rapid reaction kinetics are found in Table 3.

Dissociation Constant Determination for the S222A and D181N/S222A Mutants. Since a burst phase was observed for the S222A and D181N/S222A mutant proteins, the substrate dissociation constants were determined using values calculated for k_3. The dissociation constants for the S222A and D181N/S222A enzymes were calculated to be 0.46 and 0.81 mM, respectively (Table 3).

DISCUSSION

Several lines of evidence suggest that replacing D181 and S222 residues did not result in dramatic alterations in the tertiary structure of the enzymes. The circular dichroic spectra of the wild-type and mutant proteins are similar showing equal amounts of α-helix and β-sheet content (Figure 2). The K_i values for binding of the competitive inhibitor, phosphate, were 2.77 and 1.43 mM for the wild-type and S222A mutant, respectively (Table 2). The dissociation constants, derived from rapid reaction kinetics, for the S222A and D181N/S222A mutants were 0.46 and 0.81 mM, respectively (Table 3). Additionally, the chromatographic properties of the mutant enzymes were indistinguishable from those of the wild-type protein.

PTPases use a nucleophilic cysteine residue to attack the phosphate monoester of the substrate to form a thiol−phosphate intermediate. PTP1 has been shown to form a covalent adduct between the phosphate originating from substrate and this conserved cysteine (Guan & Dixon, 1991b). This phospho−enzyme intermediate has been trapped and characterized by ^{31}P NMR using the receptor PTPase LAR (Cho *et al.*, 1992) and observed directly during catalytic turnover using an engineered mutant of the dual specificity PTPase, VHR (Denu *et al.*, 1996a). The pK_a value of the nucleophilc thiolate in PTP1 (C215) was determined to be 5.57. This uncharacteristically low value agrees with the values of 4.6 and 5.6 for the respective cysteines found in the *Yersinia* PTPase (Zhang & Dixon, 1993) and VHR (Denu *et al.*, 1995). These values are all in sharp contrast with the apparent pK_a value of 8.3 reported for the yeast low molecular weight phosphatase Stp1 (Wu & Zhang, 1996) and free cysteine (Fersht, 1985). The crystal structures of the *Yersinia* PTPase (Stuckey *et al.*, 1994), PTP1B (Barford *et al.*, 1994), and VHR (Yuvaniyama *et al.*, 1996) suggest that the thiolate ion can be partially stabilized by a network of hydrogen bonds.

Critical amino acids involved in PTP1-catalyzed hydrolysis of *p*NPP have been analyzed using site-directed mutagenesis,

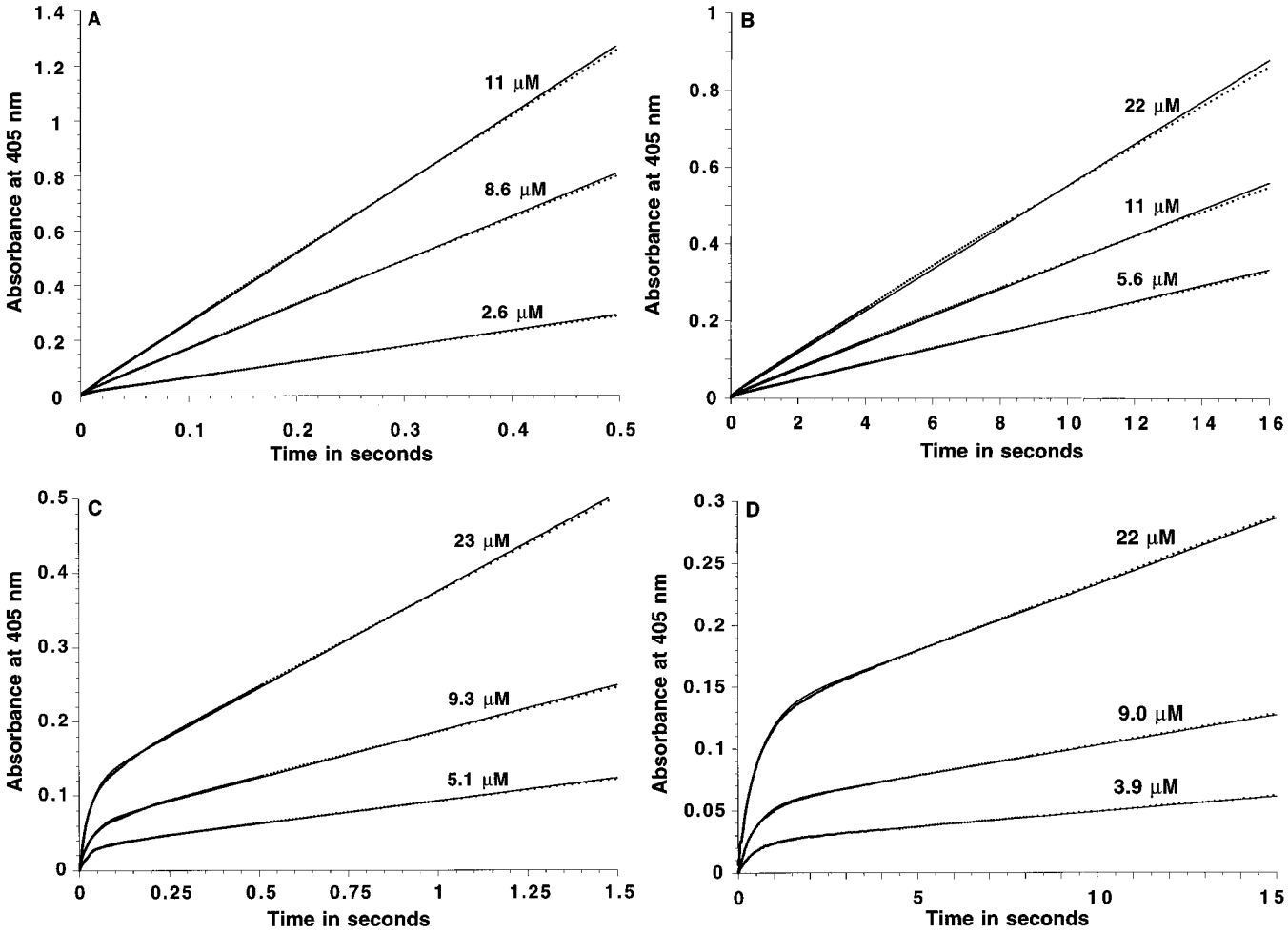


FIGURE 6: Rapid reaction kinetic traces of wild-type (A), D181N (B), S222A (C), and D181N/S222A (D) mutant phosphatases. The conditions are detailed in Materials and Methods. Each data set is the average of at least three determinations, and the solid line is the theoretical fit to eq 6. The enzyme concentrations used in each experiment are associated with each trace.

Table 3: Summary of Presteady State Kinetic Parameters for Wild-Type and Mutant PTP1 Enzymes

enzyme	conctn (μM)	burst amplitude (μM)	k_{cat} (s^{-1})	k_3 (s^{-1})	k_5 (s^{-1})	K_d (μM)
wild-type	11		20			
D181N	22		0.27			
S222A	23	13	1.3	34	1.4	0.46
D181N/S222A	22	14	0.055	1.9	0.057	0.81

chemical modification, and pH dependency of the kinetic parameters k_{cat}/K_m and k_{cat}. The pH dependency of the k_{cat}/K_m value for the wild-type enzyme indicates that there are two groups with apparent pK_a values of 5.1 and 5.44 that must be deprotonated and one group with a pK_a value of 4.93 that must be protonated for binding and/or catalysis (Figure 3 and Table 2). Zhang (1995) has done a similar study of wild-type PTP1 using *p*NPP and found pK_a values of 5.13, 5.46, and 5.06, respectively. The pK_a values obtained for VHR (Denu *et al.*, 1995) and the *Yersinia* PTPase (Zhang *et al.*, 1994b) agree with results determined in the present study. The k_{cat}/K_m pH-dependent values for VHR (Denu *et al.*, 1995) and the *Yersinia* PTPase (Zhang *et al.*, 1994b) displayed a group that must be deprotonated with a pK_a value of 5.1 indicating PTPases react with the dianionic form of substrate (*p*NPP), and therefore the pK_a value of 5.1 is observed in all k_{cat}/K_m rate profiles. The k_{cat} pH rate profile agrees very well with the results of Zhang (1995), establishing that a group with a pK_a value of 4.73 must be deprotonated and another group with a pK_a value of 6.75 must be protonated. The pH-independent value of 108 s^{-1} also agrees with the results found by Zhang (1995). There is also good evidence to support D181 as the general acid in PTP1 catalysis. If this aspartic acid residue is the general acid in PTP1, then changing it to asparagine might affect the pH rate profiles for both k_{cat}/K_m and k_{cat}. This is evident from the data presented in Figures 3 and 4 as well as in the values reported in Table 2. The D181N site-directed mutant of PTP1 exhibited a decrease of the pH-independent variable by 3 orders of magnitude in the k_{cat}/K_m rate profile. Mutagenesis of Asp-92 to Asn in the dual-specificity PTPase VHR reduces activity 2 orders of magnitude and renders the mutant enzyme pH-independent above 6.0 (Denu *et al.*, 1995). This pH independence is also seen with PTP1 suggesting that D181 is functioning as a general acid. Linear free energy relationships using the D181N mutant have shown that both the k_{cat}/K_m and k_{cat} values exhibit a large dependence on the leaving group pK_a value while the wild-type protein showed no significant dependency (Denu *et al.*, 1996a). These results suggest that the proton of aspartic acid is concomitantly transferred to the phenolic oxygen as the P−O bond is broken, neutralizing the developing negative charge. Heavy atom kinetic isotope effects have also established the contribution of D181 to the transition state for PTP1 catalysis (Hengge *et al.*, 1995). The isotope effects

have indicated that no negative charge is developed on the leaving group oxygen, consistent with the lack of any significant effects of leaving group pK_a value on the k_{cat}/K_m and k_{cat} parameters (Zhang, 1995; Hengge *et al.*, 1996; Denu *et al.*, 1996a). Collectively the observations strongly suggest that D181 and the corresponding residues in VHR (D92) and the *Yersinia* PTPase (D356) donate a proton to the leaving phenolic oxygen during phosphomonoester bond cleavage.

An additional ionization (pK_a= ~8.5) was observed in the k_{cat}/K_m pH profile for the D181N mutants. No corresponding ionization was observed in the k_{cat} pH profile, indicating that this ionization is important for efficient substrate binding. It is likely that this group is also present in the wild-type enzyme; however, its ionization is masked in the pH profile by the ionization of D181. There are no obvious residues to assign to this ionization. It is probable that this group may play a structural role at a site removed from the active site, and protonation/deprotonation affects the catalytically active conformation.

The crystal structures of the *Yersinia* PTPase, PTP1B, and VHR suggest that the hydroxyl moiety of an invariant serine or threonine residue is within hydrogen-bonding distance (2.5−3 Å) of the active site thiol (Stuckey *et al.*, 1994; Barford *et al.*, 1994; Yuvaniyama *et al.*, 1996). Changing this residue to an alanine could disrupt this proposed interaction and alter the ability of PTP1 to catalyze dephosphorylation. The k_{cat}/K_m pH rate profile of the S222A mutant is similar to that of the wild-type enzyme. The curve indicates that two ionizable groups must be deprotonated and one group must be protonated for activity. Based on the k_{cat}/K_m pH profile of the mutant enzyme, the pK_a value for C215 is 6.36 (Table 2), a full pH unit increase compared to wild-type enzyme. These results suggest that the hydroxyl group of S222 might play a role in stabilizing the low pK_a value of the active site thiol as suggested by the crystal structures. The S222A mutant exhibited greater than a 200-fold decrease in k_{cat} (Table 2). The activity of the wild-type enzyme varies over 2 orders of magnitude throughout the entire pH range analyzed, yet the sensitivity of the S222A mutant toward pH varies only 2-fold (Figure 4). The dramatic change in only the k_{cat} profile suggests that S222 is affecting some stage of the kinetic mechanism after the first irreversible step, possibly the hydrolysis of the thiophosphate intermediate.

As is evident from the data in Figure 6C, the presteady state reaction of the S222A mutant exhibited a burst of *p*-nitrophenol formation followed by a slow linear rate. The burst amplitude was approximately 70% of final enzyme concentration. The slow linear rate of 1.4 s^{-1} (Figure 6C, Table 3) is in excellent agreement with the k_{cat} value of 1.3 s^{-1} for the S222A, consistent with intermediate hydrolysis being fully rate-limiting. The steady state k_{cat} value for wild-type enzyme is 20 s^{-1}, and the rate of burst formation of the S222A mutant is 34 s^{-1}. With this mutant, the exponential rate of *p*-nitrophenol burst is expected to be the first-order rate constant (k_3, Scheme 1) for intermediate formation, assuming substrate saturation. Since we have proposed that the overall rate-limiting step in k_{cat} for the wild-type enzyme is intermediate formation, the value of k_{cat} should agree with the rate of *p*-nitrophenol formation observed in the burst phase of the reaction seen with the serine mutation. As expected, there is good agreement between these values. Given the 20-fold difference between the rates of k_3 and k_5, the 70% of predicted burst amplitude observed indicates that ~30% of the purified enzyme is inactive.

As is evident in Figure 6, no significant burst was observed when either wild-type or D181N mutant was rapidly reacted against *p*NPP. These observations are consistent with rate-limiting intermediate formation. In apparent contrast, Zhang (1995) has observed significant burst kinetics at pH 6 and 3.5 °C with a cleaved fusion protein of glutathione-*S*-transferase and $PTP1_{1-323}$. These conditions are clearly distinct from those (pH 7, 30 °C, and native $PTP1_{1-323}$) employed in the current study. Given these experimental differences, it is not unreasonable to expect discrepancies in the observations. However, taken collectively, these observations do suggest that the rates between intermediate formation and intermediate hydrolysis cannot be dramatically different such that pH and temperature can perturb the relative rates of the two steps.

To establish the possible role of D181 as the general base in PTP1-catalyzed hydrolysis of the phospho-enzyme intermediate, an S222A/D181N mutant enzyme could exploit the fact that with the S222A mutant, intermediate hydrolysis appears to be fully rate-limiting. The pH rate profile for the k_{cat}/K_m of the D181N/S222A mutant is indistinguishable from that of the D181N mutant except for an apparent shift for the pK_a value of the C215, as seen with the S222A protein (Figure 3). To see if the D181N mutation in the D181N/S222A enzyme had any effect on the rate of intermediate hydrolysis, presteady state reaction kinetics were performed. Again, as is evident from the data in Figure 6D, there was a burst of *p*-nitrophenol formation (k_3) followed by a slow linear rate (k_5). The burst amplitude was approximately 70% of final enzyme, and the slow linear rate (0.057 s^{-1}) was in agreement with the k_{cat} value (0.055 s^{-1}) and consistent with intermediate hydrolysis being rate-limiting (Table 3). When this value for k_5 is compared with the k_5 value derived from the S222A enzyme (1.4 s^{-1}), a reduction of 25-fold in catalytic efficiency is observed indicating that D181 serves an important function in intermediate hydrolysis (Table 3). It is therefore likely that D181 may act as a general base, abstracting a proton from a water molecule and facilitating intermediate hydrolysis. We have now shown that with both PTP1 and VHR (Denu *et al.*, 1996a) the D181 residue, or its equivalent, could serve as a general base in the second step of the reaction.

The amino acid sequence identity of PTP1, VHR, and the *Yersinia* PTPase is only 5% (Yuvaniyama, *et al.* 1996). Remarkably, the structures of these proteins are similar, and a careful kinetic analysis coupled with site-directed mutagenesis suggests that many features of the catalytic mechanism are highly conserved among this diverse family of catalysts.

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