The Catalytic Role of Aspartic Acid-92 in a Human Dual-Specific Protein-Tyrosine-Phosphatase†

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ABSTRACT: The mechanism of catalysis for the human dual-specific (vaccinia H1-related) protein-tyrosine-phosphatase was investigated. The pH dependence of the k_{cat} value is bell-shaped when *p*-nitrophenyl phosphate was employed as a model substrate. The k_{cat}/K_m pH profile rises with a slope of 2 and decreases with a slope of −1, indicating that two groups must be unprotonated and one group must be protonated for activity. An amino acid residue with an apparent pK_a value of 5.5 ± 0.2 must be unprotonated and a residue with a pK_a value of 5.7 must be unprotonated for activity. The pK_a value of the catalytic cysteine-124 (C124) was 5.6 ± 0.1. The aspartic acid-92-asparagine (D92N) mutant enzyme was 100-fold less active than the native enzyme and exhibited the loss of the basic limb in the pH profiles, suggesting that in the native enzyme D92 must be protonated for activity. The D92 residue is conserved throughout the entire family of dual-specific phosphatases. Mutants glutamic acid-6-glutamine, glutamic acid-32-glutamine, aspartic acid-14-asparagine, and aspartic acid-110-asparagine had less than a 2-fold effect on the kinetic parameters when compared to native enzyme. Based upon the lack of a "burst" in rapid reaction kinetics, formation of the intermediate is rate-limiting with both native and D92N mutant enzymes. In agreement with rate-limiting formation of the intermediate, the pK_a value of 5.5 for the group which must be unprotonated for activity was assigned to C124. The D92 residue acts as a general acid by protonating the phenolate ion in the rate-limiting formation of the intermediate. D92 may also serve as general base by abstracting a proton from a water molecule in the hydrolysis of the phosphoenzyme intermediate.

The dual-specific protein phosphatases are a major class of enzymes involved in mitogenic signaling and cell cycle regulation. Some members of this family (PAC1 and MKP1) appear to switch "off" mitogen-activated protein kinases (MAP kinases) (Sun *et al.*, 1993; Ward *et al.*, 1994). These phosphatases are capable of removing phosphate from both phosphotyrosine- and phosphothreonine/serine-containing proteins and peptides (Walton & Dixon, 1993). They share sequence homology to the tyrosine-specific phosphatases only at the active site motif HCxxGxxR, where the cysteine residue is required for catalysis.

It was recently demonstrated that the human dual-specific phosphatase VHR (for vaccinia H1-related)[1] containing a single amino acid substitution (C124S) at the active site would not hydrolyze either phosphotyrosine- or phosphoserine-containing substrates (Ishibashi *et al.*, 1992; Zhou *et al.*, 1994). Chemical modification of VHR by iodoacetate is highly specific, stoichiometrically modifying C124 and completely inactivating the enzyme. In addition, a covalent phosphoenzyme intermediate can be detected between VHR and the ^{32}P-labeled phosphotyrosine peptide, when the reaction is quenched under steady-state conditions (Zhou *et al.*, 1994). No adduct was observed with the C124S mutant. Taken together, these data suggested that VHR proceeds through a thiol−phosphate intermediate which is on the reaction pathway. This observation is similar to what has been observed with the tyrosine-specific phosphatases (Guan & Dixon, 1991; Cho *et al.*, 1992). Using rapid quench analysis and NMR, Cho *et al.* (1992) have trapped and characterized an intermediate formed between the tyrosine-specific protein phosphatase LAR (leukocyte antigen-related) PTPase and a peptide substrate. Additionally, a covalent adduct was observed when rat PTP1 and a ^{32}P-labeled phosphotyrosine peptide were reacted. The chemical properties of the intermediate were consistent with a thiol−phosphate linkage (Guan & Dixon, 1991).

Although there is limited sequence identity outside the conserved HCxxGxxR motif between the dual-specific and tyrosine-specific phosphatases, there is considerable sequence homology among the dual-specific phosphatases (Figure 1). Figure 1 shows a number of known dual-specific PTPases which all contain a catalytic domain of about 170 amino acids. These enzymes differ greatly both in length and in content outside this catalytic domain. VHR and VH1, 185 and 171 amino acids, respectively, appear to contain only the catalytic domain, whereas, HVH1, PAC1, and the yeast enzyme YVH1 contain additional noncatalytic domains whose functions remain unknown. Employing VHR as the model for the dual-specific protein phosphatases, we inves-

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[1] Abbreviations: PTPase, protein-tyrosine-phosphatase; 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; pNPP, *p*-nitrophenyl phosphate; D, aspartic acid; E, glutamic acid; N, asparagine; Q, glutamine; C, cysteine.

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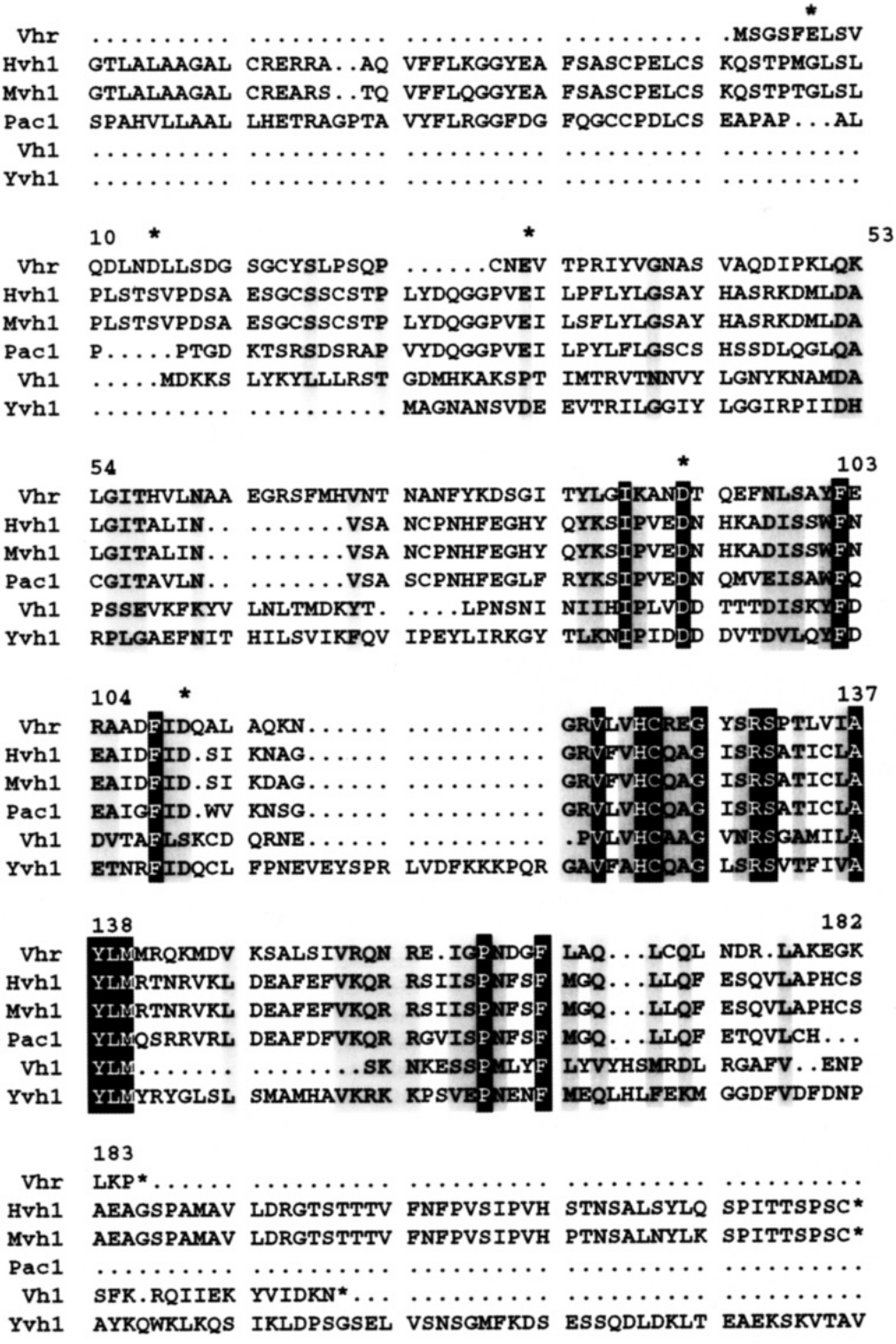

FIGURE 1: Primary sequence alignment of the dual-specific protein-tyrosine-phosphatases. Black box highlights the invariant amino acids. Gray box indicates highly conserved amino acids. The asterisks indicate the amino acids which were altered by site-directed mutagenesis. The pound symbols indicate the translation stop. The numbers refer to the 185 amino acids of VHR.

tigated the role of the conserved acidic amino acids in the catalysis of phosphate monoesters. We present a detailed investigation on the ionizations necessary for binding of substrate and catalysis using pH effects on the kinetics, site-directed mutagenesis, and chemical modification. In particular, the importance of D92, which is conserved throughout the family of dual-specific protein phosphatases, is examined. We show that D92 plays a critical role as a general acid in the catalytic mechanism and suggest a possible role for this amino acid in the breakdown of the phosphoenzyme intermediate.

MATERIALS AND METHODS

Materials. All chemicals were of the highest purity commercially available and used without further purification. The native and mutant enzymes were purified to homogeneity as judged by SDS−PAGE (Figure 2). The purification scheme employed for each mutant was as

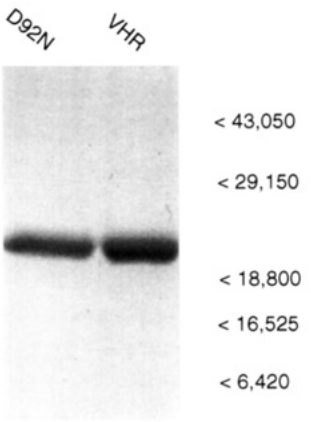

FIGURE 2: SDS−PAGE of purified native VHR and D92N mutant.

described for the native enzyme (Denu *et al.*, 1994).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out as described previously (Zhou *et al.*, 1994) using the BioRad Muta-gene method. The following oligonucleotides were synthesized in an Applied Biosystems model 391 DNA synthesizer and used to construct the indicated VHR mutants. The underlined base indicates the change from the naturally occurring nucleotides. E6Q: 5′-CACCGAGAG-CTGGAACGAGC-3′. D14N: 5′-CGAGAGCAGGTTGT-TGAGATC-3′. E32Q: 5′-GGGGTGACCTGGTTGCAGG-GC-3′. D110N: 5′-GCCAAAGCCTGGTTAATGAAGT-CG-3′. D92N: 5′-GAACTCCTGTGTGTTGTTGGCC-3′. All changes were verified by DNA sequencing.

Assays. All assays were performed using *p*-nitrophenyl phosphate (pNPP) as substrate. The buffer was a three-component system consisting of 0.1 M acetate, 0.05 M Tris [tris(hydroxymethyl)aminomethane], and 0.05 M Bis-Tris [[bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane]. This buffer maintains a constant ionic strength of 0.1 M throughout the entire pH range used in these studies (Ellis & Morrison, 1982). This prevents spurious results based upon differences in activity induced by changes in ionic strength at different pH values. The initial velocity of *p*-nitrophenol formation (E_{405nm} = 18 mM^{-1} cm^{-1}) was determined over the linear range and calculated by measuring the absorbance change after the addition of 3−20 μL of enzyme in a total volume of 0.3 mL. To determine the kinetic parameters k_{cat} and k_{cat}/K_m, the initial velocities were measured at various substrate concentrations and the data were fitted to eq 1. For the construction of the pH profiles, k_{cat} and k_{cat}/K_m were determined at various pH values. The pH data were fitted to eqs 2−5 depending upon the shape of the profile.

Data Analysis. Data were fitted to eqs 1 and 2 using the programs of Cleland (1979) and the KinetAsyst software (IntelliKinetiscs, State College, PA). Fitting of the pH-dependent data to eqs 3−5 was accomplished with nonlinear least squares fitting using NonLin for Macintosh, a Macintosh version (R. Brenstein, Southern Illinois University) of a computer code developed by Johnson and Frasier (1985). In eqs 2−5, 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.

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

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

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

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

$$v = C/[(1 + H/K_a)(1 + H/K_b)] \quad (5)$$

Inactivation of VHR by Iodoacetate. The pseudo-first-order rate constant for inactivation by iodoacetate was determined using the following method. At each pH value, five different iodoacetate concentrations (1−6 mM) were used. The inactivation was initiated by the addition of 3−20 μL of enzyme to a solution of iodoacetate and buffer at 30 °C in a total of 0.3 mL. At various times between 15 s and 30 min, 10 μL aliquots were taken and directly added to 0.3 mL of 25 mM pNPP, 100 mM acetate, pH 5.4, for activity assay. After 2 min, the reaction was quenched with 0.7 mL of 1 N NaOH and the absorbance at 405 nm was measured. The fraction of remaining activity was determined using the control reaction (no iodoacetate added) as 100%. The log value of remaining activity was plotted at each time point, and the slope of the least squares line was determined at each iodoacetate concentration. The slopes were then plotted as a function of iodoacetate concentration, and the data were fitted to a line, yielding the rate of inactivation as the slope of the line. No saturation of VHR by iodoacetate was observed.

Rapid Reaction Kinetics. Enzyme and pNPP were rapidly mixed in an Applied Photophysics stopped-flow spectrophotometer at pH 7, 30 °C. Product formation was followed at 405 nm. The slopes of the observed linear rates were determined by linear least squares fitting of the data to a best fit line. For native enzyme, substrate concentration was varied from 1 to 50 mM pNPP. With the D92N mutant, substrate concentration was varied from 0.2 to 50 mM. Equation 6 was used to calculate the predicted amplitude of the "burst" of product at various ratios of k_f/k_b, where k_f is the net rate of formation of the intermediate and k_b is the rate of breakdown (hydrolysis) of the intermediate (Fersht, 1985). The π term is the concentration of the measured product "burst", and E_0 is the enzyme concentration. To calculate the predicted absorbance change under the conditions used in these experiments, E_0 was converted to the absorbance change (E_{405nm} = 9.0 mM^{-1} cm^{-1} at pH 7) expected from a stoichiometric "burst" of product formed.

$$\pi = E_0[k_f/(k_f + k_b)]^2 \quad (6)$$

RESULTS

Identification of Critical Ionizations for the VHR-Catalyzed Reaction. The dual-specific phosphatases contain a number of conserved amino acids whose side chains might function as acid or base catalysts, a common feature of many phosphotransfer reactions (Walsh, 1979). In order 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. Because its kinetic parameters are similar to physiologically relevant phosphopeptide substrates, pNPP was employed as the model substrate for these studies (Denu *et al.*, 1994). The k_{cat}/K_m parameter is the apparent second-order rate constant for the reaction of free substrate and free enzyme and includes the binding of substrate through the first irreversible step. Ionizations which are important for binding and/or catalysis

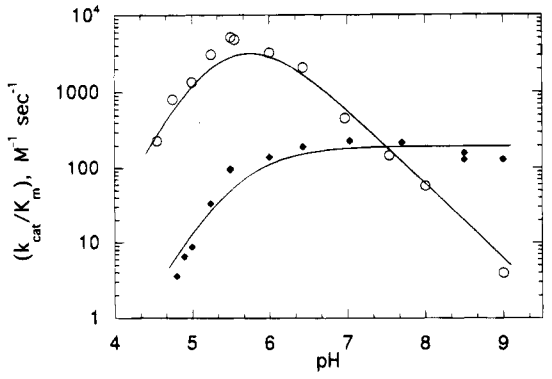

FIGURE 3: pH dependency of the k_{cat}/K_m value for native and D92N mutant VHR. The open circles represent the native enzyme data, and the solid diamonds represent the D92N mutant data. The native and D92N enzyme data were fitted to eqs 4 and 5, respectively. Results are given in Table 1. See Materials and Methods for details.

will be displayed in this pH profile. Alternatively, the pH dependency of k_{cat} will reflect the ionization state of amino acid residues involved in the rate-determining step in the overall reaction. At each pH value, the kinetic constants were determined by fitting initial velocities measured at five or more substrate concentration to the Michaelis–Menten equation (eq 1), as described in Materials and Methods. The pH profile of k_{cat}/K_m is shown in Figure 3. The plot rises with a slope of 2, reaches a plateau at near pH 5.5, and then decreases with a slope of −1, indicating that two groups must be unprotonated and one group must be protonated for activity. To determine the pK_a values of the groups involved, the data were fitted to eq 4. The results are listed in Table 1. Because of the difficulty in obtaining kinetic data at very low pH values, we cannot rule out the existence of an additional group with an extremely low pK_a value (<4.3). Two groups with pK_a values of 5.1 and 5.47 must be unprotonated while a group with a pK_a value of 5.7 must be protonated for activity. Since the pK_a values are close in value, we assumed a minimum separation of 0.6 unit between the highest and lowest pK_a values (Cleland, 1979). Thus, the pK_a values of 5.1 and 5.7 were fixed in the final analysis. Because the k_{cat}/K_m value involves the reaction of uncomplexed substrate and enzyme, the observed pK_a values must necessarily by the intrinsic pK_a values of groups on the enzyme, the substrate, or both, if substrate dissociation is faster than the rate of the chemical reaction. This is a reasonable assumption since the K_m values (>1 mM) are high. The pK_2 of pNPP is 5.1 (Zhang *et al.*, 1994a) and agrees well with the pK_a value of 5.1 observed in the k_{cat}/K_m profile. The pH-independent value of k_{cat}/K_m was 12.5 × 10^3 M^{-1} s^{-1}.

Similarly, the k_{cat} value was determined as a function of pH, and the plot is shown in Figure 4. The data indicate that two groups are involved in catalysis, one group with a pK_a value of 5.26 must be unprotonated and a group with a pK_a of 7.17 must be protonated. These pK_a values are apparent values (i.e., they are not necessarily the intrinsic values found on the free enzyme) since k_{cat} describes the reaction of the substrate–enzyme complex. The low pK_a values observed in both the k_{cat}/K_m and k_{cat} profiles suggested that acidic amino acid residues such as apartic acid and glutamic acid were playing an important role in binding and/or catalysis.

Analysis of the Conserved Acidic Amino Acids. Site-directed mutations were made in VHR at several conserved acidic amino acid residues (Figure 1) in the attempt to identify the amino acids responsible for the observed pH dependency of the two kinetic parameters. Each acidic residue was altered to the corresponding amide (i.e., E for Q or D for N) in order to minimize potential structural alterations within the recombinant protein. The kinetic parameters for the mutants D14N, D110N, E32Q, E6Q, and D92N were determined at pH 6, and the results are listed in Table 2. All the mutant enzymes except for D92N yielded k_{cat} values that ranged from 5.92 to 2.73 s^{-1} and k_{cat}/K_m values that ranged from 3240 to 1830 M^{-1} s^{-1}. Only the D92N mutation exhibited a large reduction in both k_{cat} and k_{cat}/K_m values, 87- and 23-fold at pH 6, respectively. All other changes from acidic to neutral residues resulted in less than about a 2-fold decrease compared with native enzyme. The pH dependency of the kinetic parameters of the mutant D92N was then analyzed to determine whether D92 may be responsible for one of the observed ionizations in the native enzyme. If protonation or deprotonation of D92 is required for reaction, then a change to N at this site would render the pH rate profile insensitive to pH for one of the observed native ionizations. Thus, the k_{cat} and k_{cat}/K_m values for the mutant enzyme were determined as a function of pH. Both pH profiles (Figures 3 and 4) displayed no pH dependence above pH 6 but exhibited similar behavior at acidic pH values, suggesting that D92 (observed free pK_a value of 5.7) is critical for activity and must be protonated in the native enzyme. Except for protonation of D92, the D92N mutant enzyme k_{cat}/K_m profile displayed similar values for the two remaining ionizable groups, a pK_a value of 5.1 and an amino acid residue with a pK_a value of 5.76 which must be unprotonated (Table 1).

pK_a Determination of Cysteine-124. It has been demonstrated previously (Zhou *et al.*, 1994) that C124 can be selectively carboxymethylated at low pH by iodoacetic acid, indicating that there were significant amounts of thiolate ion present. The possibility existed that the enzymatic group observed in the pH profiles with a pK_a value of about 5.6 (average) was C124. Therefore, the pK_a value of the thiol group of C124 was determined and compared to the pK_a value of approximately 5.6 observed in the k_{cat}/K_m profiles. The pK_a of C124 was determined by measuring the pseudo-first-order rate constant of inactivation of iodoacetic acid as a function of pH (Figure 5) as described in Materials and Methods. The data were fitted to eq 2 yielding a pK_a of 5.6 ± 0.1 which was in good agreement with the pK_a values of 5.47 and 5.76 observed in the native and mutant k_{cat}/K_m profiles, respectively.

Rapid Reaction Kinetics of Native and D92N Mutant. We have previously shown that a thiol–phosphate intermediate can be trapped under steady-state conditions using a ^{32}P-labeled phosphotyrosine peptide Raytide (Zhou *et al.*, 1994) and VHR. Those experiments suggested that the VHR-catalyzed reaction involves an intermediate along the reaction pathway. If the rate of breakdown of the intermediate is slower than the rate of its formation, then we would predict the appearance of a "burst" of product formation when enzyme and substrate are rapidly reacted using a stopped-flow spectrophotometer. To explore this possibility, VHR and pNPP were rapidly mixed using the stopped-flow spectrophotometer and the formation of product (pNP) at pH 7 was observed at 405 nm. No "burst" was detected with native enzyme at enzyme concentrations as high as

Table 1: Results of Kinetic Analysis[a]

enzyme	parameter units	eq	value, C	pK_1	pK_2	pK_3
native	k_{cat}/K_m	4	12.5×10^3	5.1	5.47	5.7
	(M^{-1} s^{-1})		[(9.22−16.2) × 10^3]		(5.26−5.71)	
D92N mutant	k_{cat}/K_m	5	1.96×10^2	5.1	5.76	
	(M^{-1} s^{-1})		[(1.52−2.46) × 10^2]		(5.58−5.96)	
native	k_{cat}	3	7.14		5.26	7.17
	(s^{-1})		(5.21−9.46)		(5.07−5.48)	(6.96−7.34)
D92N mutant	k_{cat}	2	5.99×10^{-2}		4.99	
	(s^{-1})		[(5.64−6.33) × 10^{-2}]		(4.90−5.08)	
native	k_{inact}	2	0.605		5.61	
	(M^{-1} s^{-1})		(0.602−0.608)		(5.50−5.72)	

[a] Confidence limits of the optimized parameters were determined using a confidence probability of 67%.

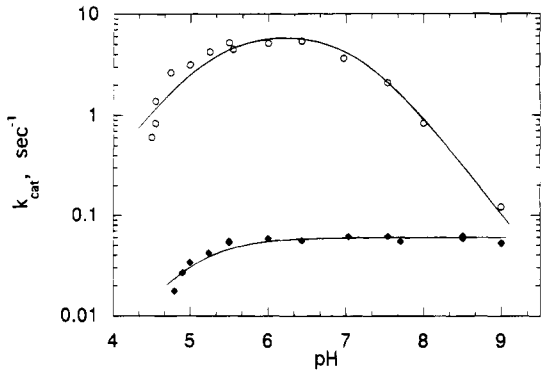

FIGURE 4: pH dependency of the k_{cat} value for native and D92N mutant VHR. The open circles represent the native enzyme data, and the solid diamonds represent the D92N mutant data. The native and D92N enzyme data were fitted to eqs 3 and 2, respectively. Results are given in Table 1. See Materials and Methods for details.

Table 2: Site-Directed Mutational Analysis of VHR[a]

enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
native VHR	5.14	1.59	3240
E6Q	5.92	2.29	2590
E32Q	4.83	1.73	2800
D110N	2.73	1.56	1755
D14N	4.85	2.65	1830
D92N	0.059	0.42	141

[a] Each data set was fitted to eq 1 as described in Materials and Methods. Standard errors were omitted for clarity. Average errors on the fitted parameters were approximately 5% and never more than 10%.

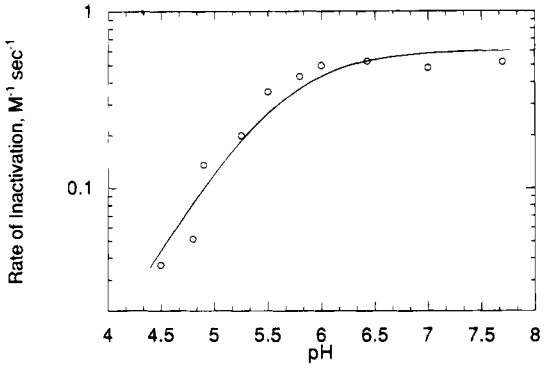

FIGURE 5: pH dependency of the rate of inactivation of VHR by iodoacetate. The data were fitted to eq 2. Results are given in Table 1. See Materials and Methods for details.

50 μM. Substrate concentration was varied to as high as 50 mM to ensure that the enzymes were saturated with substrate. Although no "burst" was observed with native enzyme, the possibility that a "burst" might be seen with the D92N mutant was also explored. If D92 is only involved in the breakdown of the intermediate and not its formation, then a "burst" might be observed using rapid kinetics. No "burst" was observed with the D92N mutant. With both enzymes, a linear rate of product formation was observed. These initial rates were determined at various substrate concentrations and then fitted to eq 1, yielding k_{cat} values of 4.44 and 0.0307 s^{-1} for native and D92N, respectively. The k_{cat}/K_m values were 545 and 16.3 M^{-1} s^{-1} for native and D92N, respectively. Both k_{cat} and k_{cat}/K_m values for native and D92N mutant are nearly identical to those measured under steady-state conditions (data shown in Figure 3), indicating that the net rate for formation was slower than the net rate for breakdown of the intermediate, such that there was no detectable amount of intermediate using this method. The apparent disparity between the rapid reaction kinetics and the previously described steady-state-trapping results can be readily explained by the fact that the ^{32}P-trapping method is extremely sensitive and can easily detect less than 1% of the total enzyme trapped as the intermediate. In the pre-steady-state reaction, if breakdown were 100-fold faster than formation, then the amplitude of the "burst" in the stopped-flow experiment would be only 0.00004 absorbance unit under the conditions of these experiments (calculated by eq 6 as described in Materials and Methods). This is obviously below the detection limits of spectrophotometric methods; however, this amount of intermediate would be detected with the radioactive trapping method. Even if breakdown were only 10-fold faster than formation, the absorbance change of the "burst" would be 0.004, which is at the limit of detection.

DISCUSSION

Enzymes can accelerate the rate of chemical reactions more than 10^8-fold over the uncatalyzed reaction (Fersht, 1985). This is often accomplished by the formation of covalent intermediates and utilizing acid/base catalysis. Covalent and acid/base catalysis are frequently encountered in mechanistic studies, and proven methodologies are available for demonstrating these strategies of rate enhancement. Detection (or trapping) of the intermediate and identification of critical ionizations by pH analysis are common means of establishing the existence of covalent and acid/base catalysis, respectively.

Both the dual-specific and tyrosine-specific protein phosphatases contain a conserved cysteine residue which is required for activity. It was shown that rat PTP1, a tyrosine-specific phosphatase, can form a covalent adduct between the phosphate originating from the substrate and the conserved cysteine (Guan & Dixon, 1991). Cho *et al.* (1992)

have trapped and characterized by ^{31}P NMR an intermediate formed between the LAR PTPase and a tyrosine-phosphorylated peptide, showing that the observed novel resonance corresponded to a thiol−phosphate. We have previously demonstrated (Zhou *et al.*, 1994) that a covalent phosphoenzyme adduct is also formed when the dual-specific phosphatase VHR reacts with ^{32}P-radiolabeled substrate under steady-state conditions. The site of linkage is thought to be at C124 since the C124S mutant enzyme is completely inactive and incapable of forming this covalent adduct. These preliminary data suggest that the dual-specific PTPases employ covalent catalysis to enhance the rate of the catalyzed hydrolysis of phosphate monoesters.

A highly reactive thiol with a low pK_a value appears to be a key feature to both the dual-specific and tyrosine-specific PTPases. With VHR, the pK_a value of the active site cysteine is 5.6 (Table 1), approximately 3 pH units lower than a normal cysteine (Fersht, 1985). Similarly, the *Yersinia* PTPase active site cysteine pK_a value is 4.6 (Zhang & Dixon, 1993). The crystal structures of the *Yersinia* PTPase and PTP1B have recently revealed that the thiolate ion is stabilized by an intricate network of hydrogen bonds to backbone amides of the catalytic loop (Stuckey *et al.*, 1994; Barford *et al.*, 1994). A similar strategy is most likely employed to lower the pK_a of the cysteine in the dual-specific PTPases.

By a combination of site-directed mutagenesis, chemical modification, and pH analysis of the kinetic parameters, we have established the important ionizations that are involved in the VHR-catalyzed hydrolysis of the phosphotyrosine analog pNPP. The pH dependency of the k_{cat}/K_m value of the native enzyme indicated that two groups with apparent pK_a values of 5.1 and 5.5 must be unprotonated and one group with a pK_a value of 5.7 must be protonated for binding and/or catalysis. The pK_a of 5.1 was not observed in the k_{cat} pH profile, suggesting that this group must be unprotonated for binding (Cleland, 1986). The pK_2 value of 5.1 for free pNPP (Zhang *et al.*, 1994a) is in agreement with this pK_a value observed in the k_{cat}/K_m pH profiles. With the *Yersinia* PTPase, Zhang *et al.* (1994a) have shown that phosphate monoesters must be in the dianionic state for catalysis. Consistent with these results, the pK_a value of 5.1 observed in the k_{cat}/K_m profiles results from the pK_2 ionization of pNPP.

Site-directed mutants of VHR were constructed and their kinetic constants analyzed to establish the specific amino acids responsible for the remaining ionizations of the pH profiles. Homogeneous preparations of the VHR site-directed mutants E6Q, E32Q, D14N, and D110N had less than a 2-fold effect on the kinetic parameters when compared to native enzyme. However, the D92N mutant exhibited about a 100-fold decrease in both k_{cat} and k_{cat}/K_m (when comparing the pH-independent values; Table 1), thus establishing the importance of this residue in the reaction. In the mutant k_{cat}/K_m pH profile, loss of a protonated group with a pK_a value of 5.7 strongly suggests that D92 must be protonated for activity in the native enzyme. The fact that the k_{cat}/K_m pH profiles of native and mutant enzyme intersect at high pH suggests that the ionizing group in the native enzyme is a carboxyl group. Ionization of this group produces a negative charge at the active site which prevents the reaction. A neutral substitution at this site (D92N) slows the reaction without preventing it. The D92 residue is also the only acidic amino acid that is conserved throughout the entire family of dual-specific phosphatases (Figure 1).

Because the loss of the basic limb in the profiles and the magnitude of the effect of the D92N mutation on *both* k_{cat} and k_{cat}/K_m were very similar, this suggests that the same rate-determining step is being observed in both parameters. The k_{cat}/K_m value involves binding of substrate through the first irreversible step in the overall reaction. Presumably, the first irreversible step would be the release of *p*-nitrophenol. As a result, k_{cat}/K_m would only involve the steps leading to formation of the intermediate and release of *p*-nitrophenol and would not include the breakdown of the thiol−phosphate intermediate. Moreover, the inability to observe a "burst" of product formation using stopped-flow kinetic techniques is consistent with rate-limiting formation of the intermediate. However, since we can trap a small fraction of ^{32}P-labeled enzyme with labeled phosphotyrosine peptide (Zhou *et al.*, 1994), the net rate of formation of the intermediate cannot be negligible relative to the net rate of breakdown. Rate-limiting intermediate formation is consistent with the observed ionizations of the k_{cat}/K_m pH profile. The pK_a value of 5.6 for C124 determined from chemical modification studies is in good agreement with the value of 5.6 (average) observed in the k_{cat}/K_m pH profiles, suggesting that the thiolate anion is involved in the rate-limiting step. The nucleophilic thiolate ion which attacks the phosphorus atom would be expected to be observed if formation of the thiol−phosphate intermediate was rate-determining. The role for D92 would logically be to donate a proton to the leaving phenolate ion.

We evaluated the notion that because D92 protonates the *p*-nitrophenolate ion as it is formed, no "burst" would be predicted for the native enzyme (since only the *p*-nitrophenolate ion is chromophoric). There are two logical explanations for the lack of a "burst". (1) If the chemical formation of the intermediate were rate-limiting, no "burst" is expected. (2) Similarly, if chemical formation of the intermediate were fast followed by slow dissociation of *p*-nitrophenol from enzyme, no "burst" is predicted. Even though D92 protonates the *p*-nitrophenolate ion, a "burst" would be predicted if the breakdown of the intermediate were slow relative to its formation. Once off the enzyme, *p*-nitrophenol is rapidly (5×10^{10} M^{-1} s^{-1}; Eigen, 1967) converted to *p*-nitrophenolate (by OH^-) which can be observed as a product "burst". The second explanation for the lack of a "burst" is unlikely since *p*-nitrophenol is a very poor inhibitor of PTPases (Zhang *et al.*, 1994a), suggesting that dissociation of *p*-nitrophenol from the active site is rapid. Also, the observed ionizations from the k_{cat}/K_m pH profile are consistent with rate-limiting chemistry and not rate-limiting product release.

On the basis of the kinetic behavior of the D92N mutant enzyme compared with the native, D92 must be protonated for efficient rate-limiting formation of the intermediate. Accordingly, the group with a pK_a value of 5.3 which must be unprotonated is C124, and the group with a pK_a of 7.2 which must be protonated in the rate-limiting step (k_{cat}) is D92. These pK_a values are apparent values since they arise from the substrate−enzyme complex. Upon binding substrate, the pK_a of D92 is shifted from 5.7 to 7.2. A similar result was observed with the enzyme fumarase (Brandt *et al.*, 1963). The pK_a of the catalytic carboxyl on fumarase increases by 0.5−1.1 pH units upon binding either malate or fumarate. Also, the pK_a of the catalytic imidazole on

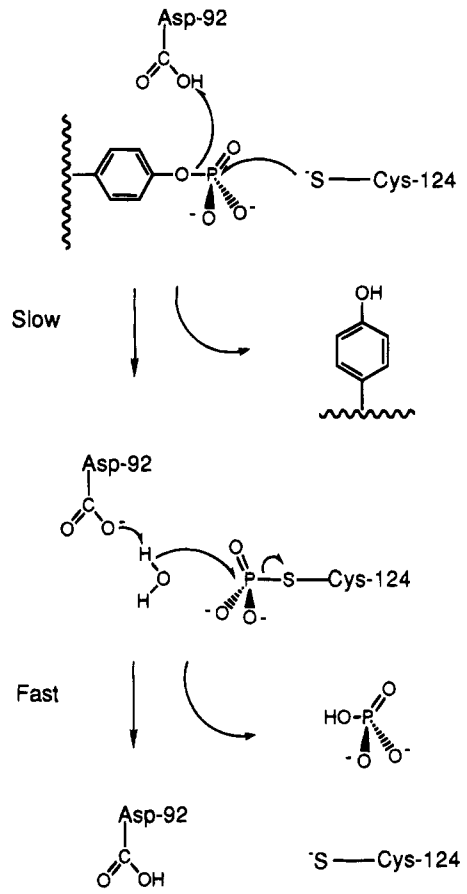

FIGURE 6: Proposed catalytic mechanism for the hydrolysis of phosphate monoesters by the dual-specific phosphatases.

fumarase shifts from 7.1 to 9 when malate binds and from 7.1 to 4.9 when fumarate binds. The shift in pK_a was the result of hydrogen bond formation between the enzyme and substrate.

From structural studies of the native *Yersinia* PTPase, Stuckey *et al.* (1994) have demonstrated that the catalytically important D356 is approximately 12 Å from the active site. The D356 residue has been suggested to function as a general acid (Zhang *et al.*, 1994), in a manner similar to that proposed for D92 in the dual-specific phosphatases. When tungstate is bound to the enzyme, there is a dramatic conformational change in a loop where D356 is located. The D356 moves approximately 7 Å toward the active site in the tungstate complex. It is quite possible that a similar conformational change brings D92 into a position where it can form a hydrogen bond to the phenolic oxygen, shifting the pK_a from 5.7 to 7.2.

Consistent with the results presented in this investigation, we propose the mechanism shown in Figure 6 for the VHR-catalyzed hydrolysis of phosphate monoesters. The phosphotyrosine dianion binds enzyme to form the binary enzyme–substrate complex. The nucleophilic thiolate anion then attacks the phosphate to form the thiol–phospho intermediate, while the D92 acts as a general acid to donate a proton to the leaving phenolate ion. There is considerable evidence to support the importance of protonating the departing oxygen as a method of catalysis for the hydrolysis of phosphate esters (Herschlag & Jencks, 1989). In phosphoryl-transfer reactions, the phosphorus–oxygen bond of the monoester is almost completely broken before addition of the nucleophile (Herschlag & Jencks, 1989). As a result there is a corresponding charge development on the departing oxygen which can be stabilized by proton transfer from a general acid. The departing leaving group and the nucleophilic cysteine residue attacking the phosphorus center lead to formation of a covalent phosphoenzyme intermediate. For hydrolysis of the intermediate, D92 now in its unprotonated form can act as a general base, abstracting a proton from a water molecule which attacks the phosphorus atom to eliminate phosphate and regenerate the active enzyme. The resulting enzyme with protonated D92 and unprotonated C124 is now poised for another round of catalysis.

Evidence in support of this mechanism comes from results obtained with the *Yersinia* PTPase, where Zhang *et al.* (1994b) have demonstrated the importance of two acidic amino acids, D356 and E290, in the enzyme-catalyzed hydrolysis of pNPP. By site-directed mutagenesis and pH kinetic analysis, it was shown that D356 must be protonated and E290 must be unprotonated for activity. These two acidic residues are conserved throughout the entire family of tyrosine-specific protein phosphatases (Zhang *et al.*, 1994b). On the basis of the crystal structure of the *Yersinia* PTPase and PTP1B (Stuckey *et al.*, 1994; Barford *et al.*, 1994), the conserved aspartic acid is positioned such that it can act as a general acid whereas the conserved glutamic acid, originally thought as a general base, forms an important ionic interaction with the conserved arginine. It is curious to note that no such corresponding glutamatic acid has yet been identified with the dual-specific PTPases. The absence of this glutamic acid may account in part for the lower reactivity of the dual-specific phosphatases. Nevertheless, it does appear that D356 in the *Yersinia* PTPase and D92 in VHR are playing similar roles. Zhang *et al.* (1994b) have proposed that D356 is acting as a general acid in the hydrolysis of phosphate monoesters.

Recent work on a class of enzymes referred to as low molecular weight acid phosphatases has revealed many similarities with the PTPases and the dual-specific PTPases. These enzymes share the conserved cysteine and arginine (CxxxxxR) with the PTPases but exhibit no apparent sequence identity outside this region. The crystal structures of two bovine low molecular weight acid phosphatases have implicated D129 as a potential general acid in catalysis (Su *et al.*, 1994; Zhang *et al.*, 1994). Recent mutational studies on the (bovine liver) low molecular weight acid phosphatase provided evidence for the importance of D129 in catalysis (Taddei *et al.*, 1994). These three major classes of protein tyrosine phosphatases share no sequence homology outside the short stretch of seven amino acids, but they all appear to employ an aspartic acid as an important catalytic residue. The catalytic loop consisting of an essential cysteine and arginine and a catalytically critical aspartic acid located on a different loop are a common evolutionary solution to catalysis from diverse protein tyrosine phosphatases (Barford *et al.*, 1994; Stuckey *et al.*, 1994; Su *et al.*, 1994; Zhang *et al.*, 1994).

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