Mutational and Kinetic Evaluation of Conserved His-123 in Dual Specificity Protein-tyrosine Phosphatase Vaccinia H1-related Phosphatase

PARTICIPATION OF TYR-78 AND THR-73 RESIDUES IN TUNING THE ORIENTATION OF HIS-123*

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Active-site cysteine strategically positioned in the P-loop of protein-tyrosine phosphatases has been suggested to be further stabilized by hydrogen bonding arrays radiating out from the P-loop to neighboring residues. In this work, we investigated the structural role of histidine array in HC(X)₅RS motif of the vaccinia H1-related protein phosphatase (VHR), using site-directed mutagenesis in conjunction with an extensive kinetic analysis. Conserved His-123 was mutated along with neighboring residues Tyr-78 and Thr-73. The increased pKₐ values of active-site Cys-124 found in Y78F and T73A mutants (6.51 and 6.75, respectively) were comparable to those of H123A and H120F mutants. Kinetic evaluation of Y78F and T73A mutants further implicated that the mutations perturb the relative position of Cys-124 within the P-loop. These results imply that Tyr-78 and Thr-73 make up an essential part of the His-123 array and structurally tune the Cys-124 position. Tyr-78 of VHR turns out to be the invariant Tyr reported in several protein-tyrosine phosphatases by a structure-based sequence alignment. Therefore, orientation of the imidazole ring of His-123 by the invariant Tyr-78 is crucial for maintaining the proper position of Cys-124 in the P-loop.

The superfamily of protein-tyrosine phosphatases (PTPs) consists of a diverse group of enzymes that shares the signature motif of Cys-(X)₅-Arg (1). The PTPs in conjunction with protein-tyrosine kinases regulate cell growth, differentiation, and proliferation by controlling the overall level of cellular tyrosine phosphorylation. Recent crystal structure determinations of several types of PTPs made elucidation of reaction mechanisms of PTPs possible (2, 3). Numerous three-dimensional structures of catalytic domains of PTPs have been resolved including Yersinia PTP (4), low molecular weight PTP (5), PTP1B (6), VHR (7), PTPα (8), SHP-1 (9), and Cdc25A (10).

The reaction mechanism of vaccinia H1-related phosphatase (VHR), which belongs to the dual-specific protein phosphatase (11–13), has been extensively studied (14, 15). Recently, VHR has been identified as a tyrosine-specific extracellular signal-regulated kinase phosphatase (16). Like other PTPs the active-site Cys-124 is stabilized by an extensive network of hydrogen bonding between backbone NH of the P-loop and the thiolate anion (4, 17, 18). The sulphydryl group of Cys-124 has an unusually low pKₐ value and functions as a nucleophile to form a thio-phosphate-enzyme intermediate (19). Asp-92 serves as a general acid catalyst for formation of the phosphoenzyme intermediate (20), and in the hydrolysis of thio-phosphate intermediate the same Asp-92 acts as a general base, and the hydroxyl group of Ser-131 is transiently linked to the thiol (14) (Scheme 1). The phosphoenzyme intermediate of VHR has been verified by ³¹P NMR (21), and the structure of the transition state forming the intermediate has been suggested to be a highly dissociative metaphosphate-like transition state (22). Additionally, by using a variety of nonpeptide substrates, the decomposition of phosphoenzyme intermediate has been shown to be the rate-limiting step for the VHR-catalyzed reaction (15).

The crystal structure of VHR further explains some catalytic properties of VHR (7). The shallow active-site pocket in VHR is likely to be related to the broad substrate specificity of VHR, which is known to be favorable toward sterically demanding nonpeptidic substrates (23). The absence of a water molecule in the cavity of VHR coincides with a nucleophilic reactivity of alcohols observed in VHR (24). The Glu-450 of Yersinia PTP ligates a water molecule and effectively prevents nucleophilic attack of alcohol from outside the cavity. The VHR structure also reveals that the position of conserved Asp-92 is different from the flexible WPD-loop found in Yersinia PTP or PTP1B (3). However, there are some structural features of VHR that have not been adequately recognized (7). One is that imidazole N-δ of His-123 forms a hydrogen bond with the carbonyl oxygen of Cys-124. This imidazole ring of VHR is further connected to a water molecule held between Tyr-78 and Thr-73 by hydrogen bonds (see Fig. 1). The water molecule ligated at this position seems to be unique to VHR. His-123 of VHR is known to be conserved in most PTPs as HC(X)₅R(S/T) except low molecular weight PTPs where His is replaced by Val (17). However, studies on the function of the conserved His are surprisingly sparse.

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§ The abbreviations used are: PTP, protein-tyrosine phosphatase; VHR, vaccinia H1-related phosphatase; Bis-Tris, bis(2-hydroxyethyl)imidolizine-1-(2-hydroxyethyl)ethane; pNPP, para-nitrophenyl phosphate; pTyr, phosphotyrosine; pThr, phosphothreonine; PMP, phenethylamine monophosphate; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography.
One early report on the role of His-402 of Yersinia PTP shows that the His residue is critical for stability of the active-site Cys-403 (25). When His-402 was substituted with Asn or Ala, the pK_a of the active-site Cys-403 increased dramatically. The authors, not knowing the structure of Yersinia PTP at that time, proposed a direct interaction between the imidazole ring of His-402 and thiol of Cys-403. This proposal is not compatible with the His position in the P-loop of Yersinia PTP determined by X-ray crystallography (4). Now all conserved His residues in VHR, PTTP1B, and Cdc25A appear to interact with the carboxyl group of the active-site cysteine residue by a hydrogen bond.

In this study, to verify the structural role of the conserved His, we first carried out site-directed mutagenesis on His-123 of VHR, and we examined its effect on pK_a of the active-site Cys-124. Then we examined the possible role of the hydrogen bonding array involving the conserved His-123, Tyr-78, and Thr-73 in orienting Cys-124 at the P-loop for optimal catalytic activity by mutating Tyr-78 and Thr-73. Extensive kinetic analysis was carried out with mutants of Y78F and T73A to demonstrate that the hydrogen bonding array of His-123 is crucial for maintaining the proper positioning of Cys-124 in the catalytic site.

EXPERIMENTAL PROCEDURES

Materials—The substrate materials para-nitrophenyl phosphate, phosphotyrosine, phosphothreonine, and phenylphosphate monophosphate were purchased from Sigma. The sequencing grade endoproteinase Lys-C and microcentrifuge filter with molecular cut-off of 10,000 were also obtained from Sigma. Malachite green oxalate was purchased from Junsei (Tokyo, Japan), and ammonium molydate was from Shinnyo (Osaka, Japan). pET21a vector for bacterial expression was obtained from Novagen (Madison, WI), and isopropyl-thio-galactoside was obtained from Roche Molecular Biochemicals. Hitrap Heparin and Superose 12 HPLC column were purchased from Amersham Pharmacia Biotech. [3H]Iodoacetic acid was obtained from PerkinElmer Life Sciences.

Site-directed Mutagenesis—Substitutions of residues of Thr-73, Tyr-78, Asp-92, and His-123 of VHR were carried out by the site-directed mutagenesis procedure of Cormack et al. (26). The oligonucleotide primers used were as follows: T73A, ATGACAGCTCAAGCCATGCAAC; Y78F, ACACACAGCCTTCACTGCTCCGCA; D92N, ATCCAACTGCAATCTGACCG; H123A, GCGGTGCTGCGTCTGCGTCTGCGGAGG; T73A, ATGCACGTCAACGCCAATGCCAAC; D92N, ATCCAACTGCAATCTGACCG; H123A, GCGGTGCTGCGTCTGCGTCTGCGGAGG; T73A, ATGCACGTCAACGCCAATGCCAAC; D92N, ATCCAACTGCAATCTGACCG; H123A, GCGGTGCTGCGTCTGCGTCTGCGGAGG; T73A, ATGCACGTCAACGCCAATGCCAAC; D92N, ATCCAACTGCAATCTGACCG; H123A, GCGGTGCTGCGTCTGCGTCTGCGGAGG; T73A, ATGCACGTCAACGCCAATGCCAAC; D92N, ATCCAACTGCAATCTGACCG; H123A, GCGGTGCTGCGTCTGCGTCTGCGGAGG. All of the mutants were verified by DNA sequencing.

Expression and Purification—The wild-type and mutant VHRs were cloned in pET21a vector and transformed to Escherichia coli strain BL21(DE3) for protein expression. The overexpression and purification of VHR were performed according to Zhou et al. (19) with some modifications. The pre-chromatographic steps were the same as reported previously (19) except that Hitrap Heparin and Superose 12 HPLC chromatography were used instead of S-Sepharose and Sephadex G-75. The pre-chromatographic steps were the same as reported previously (19) except that Hitrap Heparin and Superose 12 HPLC chromatography were used instead of S-Sepharose and Sephadex G-75. The buffer system containing 0.1 M acetate, 0.05 M Tris, and 0.05 M Bis-Tris at a constant ionic strength of 11500 mM was used (28).

Determination of pK_a of Cys-124—For determination of pK_a of wild-type and mutant VHRs, a direct 3H-carboxymethylation method was used (20, 25). A portion of 2 μg of enzyme in each pH value was incubated with 5 μCi of [3H]iodoacetic acid (344.2 mCi/mmol) for 100 min at 30 °C. The labeled protein was precipitated with 10% trichloroacetic acid on ice and washed three times with ice-chilled trichloroacetic acid, and the radioactivity bound on protein was measured as a function of pH and fitted to the equation

\[
\text{pK}_a = \frac{k_{bt} (1 + H/K_a)}{k_{bt} (1 + H/K_a)}
\]

Here H and K_a are the proton concentration and the acid dissociation constant of the Cys-124, respectively. In order to verify this method, peptide mapping was performed with T73A mutant as a representative. After carboxymethylation by [3H]iodoacetic acid with 15 μg of T73A at each pH, the labeled protein was divided into two portions as follows: one for measuring the total amount of bound radioactivity, and the other for digestion with 0.2 μg of Lys-C endoproteinase. The reversed phase chromatography of digested protein was done with Vydac C18 column (4.6 × 250 mm, 300-A pore size, 5-μm particle size) at a flow rate of 0.5 ml/min. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 80% acetonitrile in solvent A. The percentage of solvent B increased from 0 to 70% during the 40-min linear gradient. The radioactivity of each fraction was counted, and the composition of amino acid was determined. The Cys-124 was found on the major radioactive peak (fragment of NGRVLHCRYGSSRPT) eluted at 24 min. The radioactivity counts from total protein and the counts from peptide fraction containing Cys-124 were simultaneously plotted as a function of pH and fitted to the above equation. To confirm the pK_a values for Y78F and Y78F mutant and purified by the same method, and the purity of isolated wild-type and mutant VHRs was checked on SDS-polyacrylamide gel electrophoresis.

VHR Phosphatase Assay—VHR assay was performed at 30 °C as described by Denu and Dixon (14). The buffer system containing 0.1 mM acetyl, 0.05 mM Tris, and 0.05 mM Bis-Tris at a constant ionic strength of 0.1 mM was used throughout the entire pH range. Nonpeptide phosphate esters, pNPP, pTyr, pThr, and PMP, were used as substrates. When pNPP was used, the phosphatase activity was measured by absorbance at 405 nm after quenching with 0.5 mM NaOH. In the case of pTyr, pThr, and PMP, the inorganic phosphate released was measured by colorimetry at 660 nm using malachite green solution (27). For obtaining Michaelis-Menten parameters, k_cat and k_cat/K_m, the initial velocities were measured at eight different substrate concentrations between 0.1 and 5 K_m. The initial velocities were fitted to the Michaelis-Menten equation using the nonlinear data analysis program (Axum, Mathsoft, Inc.). The enzyme concentration was determined from absorbance at 280 nm using an extinction coefficient of 11500 absorbance units per mg per cm.
iodoacetate (Table I). The carboxymethylation of cysteine by iodoacetate has been successfully tested in Yersinia PTP (25) and VHR (19). The \( pK_a \) values were estimated from the radioactivity bound to proteins at different pH values (Fig. 2). The \( pK_a \) of 5.52 (5.44–5.62) estimated for wild-type VHR agrees well with the reported value of 5.6 ± 0.1 (20). The \( pK_a \) values of His-123 mutants, H123A and H123F, were 6.88 (6.75–7.07) and 6.93 (6.82–7.08), respectively. These values are at least 1.3 pH units higher than that of the wild-type VHR. The shift of \( pK_a \) value of the active-site cysteine by eliminating the hydrogen bonding capacity of His-123 could be compared with the \( pK_a \) shift reported on His-402 mutants of Yersinia PTP (25). However, the H123N mutant, which still forms hydrogen bonding with neighboring residues, showed a similar \( pK_a \) value of 5.47 (5.37–5.58) compared with that of the wild-type enzyme.

The second series of mutants, Y78F and T73A, were designed to investigate the role of amino acids around His-123. The \( pK_a \) values of the active-site cysteine in mutants, Y78F and T73A, were found to be 6.51 (6.38–6.69) and 6.75 (6.68–6.90), respectively. These results are intriguing, because these mutants disturb only the hydrogen bond connected to the N-ε side of imidazole ring of His-123 via a water molecule, and it is not apparent how the distant hydrogen bonds would affect the active-site cysteine (Fig. 1). To confirm further the \( pK_a \) value obtained, we carried out peptide mapping for \(^3\)H-carboxymethylation of Cys-124 separated by peptide mapping.

**Effect of pH on Kinetic Parameters of Y78F and T73A Mutants—**Observation of the marked alterations in kinetic parameters obtained with Y78F and T73A mutants led us to investigate the pH dependence of the kinetic parameters using pTyr as a substrate (Fig. 3). Determination of kinetic parameters, \( k_{cat} \) and \( K_m \), as a function of pH could establish the residues that function as acid or base catalysts (30). The \( k_{cat}/K_m \) parameter is the apparent second order rate constant for the reaction of substrate and free enzyme. Therefore, in the pH–\( k_{cat}/K_m \) profile, ionization residues that are important for binding of substrate and/or catalysis of the first irreversible step would be displayed, and the pH–\( k_{cat}/K_m \) profile would display the ionization state of residues involved in the rate-limiting step in the overall reaction. The pH–\( k_{cat}/K_m \) profiles for wild-type, Y78F, and T73A displaying bell shapes, with an ascending slope of approximately +2 and a descending slope of approximately −1, were observed (Fig. 3A). These are mostly consistent with the known participations of one unprotonated residue together with the dianion of phosphate monoester for the ascending curve and a protonated residue for the descending curve during the VHR-catalyzed formation of phosphoenzyme intermediate (20). However, there was a small but significant shift in optimal pH of the mutants toward alkali compared with that of the wild type. When \( k_{cat} \) was plotted against pH, the mutants showed a more drastic difference from

### Table I

**\( pK_a \) values of Cys-124 in wild-type and mutant VHRs**

| Enzyme    | \( pK_a \) |
|-----------|------------|
| Wild type | 5.52 (5.44–5.62) |
| H123A     | 6.88 (6.75–7.07) |
| H123F     | 6.93 (6.82–7.08) |
| H123N     | 5.47 (5.37–5.58) |
| Y78F      | 6.51 (6.38–6.69) |
| T73A      | 6.75 (6.68–6.90) |
| T73A/Y78F | 7.04 (6.94–7.15) |

\( * \) \( pK_a \) value was estimated from pseudo-first order inactivation rate constant.

\( * \) \( pK_a \) value was determined by measuring the radioactivity of \(^3\)H-carboxymethylated Cys-124 separated by peptide mapping.
that of the wild-type enzyme (Fig. 3B). The descending part (alkaline wing) of the normal bell shape disappeared in the mutants. Since the alkaline wing reflects the participation of protonated residue in the rate-limiting step, these $k_{cat}$ alterations of mutants are likely due to shift of the ionization state of protonated residue(s) involved in the breakdown of phosphoenzyme intermediate (Scheme 1).

The $pK_a$ values of ionizable group involved in the pH dependence data of pTyr substrate were analyzed, and the results are listed in Table III. For wild-type VHR, the analysis of $k_{cat}/K_m$ shows $pK_a$ values of 5.61 ($pK_1$) and 5.43 ($pK_2$) as the two unprotonated species, whereas a $pK_a$ value of 5.71 ($pK_3$) seems to reflect the protonated residue. Here the $pK_1$ of 5.61 should be the unprotonated dianion of substrate pTyr ($pK_{d2}$ of pTyr). The $pK_2$ of 5.43, another unprotonated group calculated from $pH-k_{cat}/K_m$ profile, seems to correspond to the $pK_a$ of Cys-124. The value of $pK_2$ is in good agreement with the $pK_a$ of Cys-124 determined by iodoacetate titration (Table I). The most probable candidate for protonated residue ($pK_3$) could be the Asp-92, since Asp-92 acts as a general acid catalyst for formation of the phosphoenzyme intermediate. These $pK_a$ values ($pK_1$, $pK_2$, and $pK_3$) values obtained with pTyr substrate are basically similar to those derived from pNPP hydrolysis except the $pK_1$ ($pK_{d2}$ of pTyr) (20). For mutants $k_{cat}/K_m$, the analysis shows that both $pK_a$ and $pK_c$ apparently increased by 1–1.7 and 0.3–0.7 $pK$ units, respectively, indicating that the mutations affect unprotonated and protonated states of residues involved in substrate binding and/or catalytic step ($k_c$) for formation of phosphoenzyme intermediate. The analysis of pH-dependent $k_{cat}$ values of wild-type enzyme indicates that one residue with a $pK_a$ value of 5.52 ($pK_2$) should be unprotonated and the other with a $pK_a$ value of 7.02 ($pK_3$) should be protonated in the rate-limiting step, likely the breakdown of phosphoenzyme intermediate in the case of substrate pTyr. The $pK_a$ components of $k_{cat}$ of mutants have not been observed as evidenced by the absence of a descending curve in the alkaline pH studied here. When the kinetics were carried out with PMP as a substrate, a substantially diminished effect of pH on $k_{cat}/K_m$ was observed (Fig. 3C). For mutants, the pH dependence of $k_{cat}/K_m$ values were further reduced.

**Substrate Specificity of T78F and T73A Mutants**—A series of studies on substrate specificity of VHR has established that VHR catalyzes hydrolysis of aromatic phosphate as well as alky phosphate, although the former is preferred (15, 23, 28). Since kinetic parameters of Y78F and T73A mutants determined with pTyr substrate reveal substantial changes from those of wild-type VHR, we extended our kinetic studies to different types of nonpeptide substrates (Table IV). Effectively similar $k_{cat}$ values for each wild-type or mutant VHRs were obtained with different substrates at pH 6.0 as expected by the fact that the breakdown of phosphoenzyme intermediate is the rate-limiting step for VHR-catalyzed hydrolysis of phosphate monoesters (15). However, there was a rather big discrepancy between $k_{cat}$ values for pNPP and pThr at pH 7.0. Like $k_{cat}/K_m$ for Y78F and T73A measured using pTyr as substrate at pH 6.0 (Table II), the $k_{cat}/K_m$ of mutants obtained with pNPP and pThr showed also a substantial reduction (8–13-fold for Y78F and 3–5-fold for T73A) as expected by the kinetic parameters of Y78F and T73A mutants. The $k_{cat}/K_m$ values obtained for each substrate are more evident when the ratios of $k_{cat}/K_m$ values observed with PMP as substrate show a rather small decrease (3–5-fold). The effects of substrates on $k_{cat}/K_m$ are more evident when the ratios of $k_{cat}/K_m$ values obtained with different substrates are compared with each other among the mutants. The 11-fold difference of $k_{cat}/K_m$ values observed between PMP and pNPP in wild-type VHR jumps to a 120-fold difference in the T73A mutant at pH 6.0. The $K_v$ values obtained for each substrate are consistent with the results of substrate effect on $k_{cat}/K_m$ values. All substrates examined except PMP showed increased $K_v$ values upon mutation. The unaffected $K_v$ values for PMP in mutants, together with the smallest $K_v$ value observed with PMP, suggest that the active site of VHR tends to be easily occupied by a large hydrophobic substrate.

**Effect of Mutation on Turnover Rate**—To ascertain the rate-limiting step of wild-type and Y78F mutant, pre-steady-state

### Table II

**Kinetic parameters of wild-type and mutant VHRs using phosphotyrosine as a substrate at 30 °C**

| Enzyme     | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ |
|------------|-----------|---------------|-------|
| Wild type  | 3.08 ± 0.34 | 835 ± 26 | 3.69 ± 0.42 |
| H123A      | 0.30 ± 0.03  | 278 ± 26.6  | 10.7 ± 1.39  |
| H123F      | 0.11 ± 0.01  | 42.9 ± 0.23  | 2.50 ± 0.21  |
| H123N      | 0.84 ± 0.14  | 714 ± 23    | 1.17 ± 0.043 |
| Y78F       | 0.07 ± 0.03  | 79.1 ± 8.0   | 9.10 ± 1.25  |
| T73A       | 0.22 ± 0.01  | 23.2 ± 2.4   | 9.36 ± 1.04  |
| T73A/Y78F  | 0.39 ± 0.11  | 1.75 ± 0.02  | 226 ± 65.9   |
| D92N       | 0.026 ± 0.006 | 15.6 ± 0.15  | 1.67 ± 0.039  |

### Figure 2

**Determination of $pK_a$ of Cys-124 in wild-type and mutants VHRs.**

A. $pK_a$ value of Cys-124 determined by $\text{H-carboxymethylation}$ method. The counts from protein-bound radioactivities were fitted with nonlinear regression to $k_{cat} = k_{lim}/(1 + H/K_a)$. Here $H$ and $K_a$ are the proton concentration and acid dissociation constants of the Cys-124, respectively. This equation can be rearranged to $k_{lim}/k_{cat} = K_a / (H + K_a)$ for the dissociated fraction of Cys-124.

B. Verification of the carboxymethylation method by peptide mapping of T73A.

C. Determination of the $pK_a$ value of Cys-124 for Y78F by pseudo-first order rate constant of inactivation by iodoacetic acid. The $k_{lim}$ value was estimated to be 0.87 M⁻¹ s⁻¹. Three independent experiments were conducted.

![Figure 2](http://www.jbc.org/doi-fig)
The kinetic experiments were carried out with 20 mM pNPP at 30 °C (Fig. 4). The burst kinetic traces observed with both VHRs apparently revealed the rate-limiting step of Y78F was the breakdown of the phosphoenzyme intermediate. From kinetic experiments the values of $k_{3}$ were consistently smaller than the values of $k_{2}$. Although the $k_{3}$ value diminished significantly by mutation, the $k_{2}$ values were virtually unchanged. This result indicates that the effect of mutation mainly affects the $k_{2}$, the turn-over rate ($k_{cat}$). For further examination of the $k_{3}$ slow-down effect by mutation, we examined the effect of ethylene glycol on the $k_{cat}$ of wild-type and mutant VHR proteins with various non-peptide substrates.

**Table III**

Analysis of kinetic parameters for wild-type and mutant VHR proteins with pTyr as a substrate

Data were fit to Equations 1–3 for each parameter using the nonlinear data analysis program Axum. Equation 1, $k_{cat}/K_{m} = (k_{cat}/K_{m})_{lim}(1 + H/K_{a})(1 + H/K_{b})$, Equation 2, $k_{cat} = k_{cat}^{lim} / (1 + H/K_{a})$, Equation 3, $k_{cat} = k_{cat}^{lim} / (1 + H/K_{b})$. In equations, $H$ is the proton concentration, $K_{a}$, $K_{b}$, and $K_{c}$ are the ionization constants of the groups involved in the reaction. $(k_{cat}/K_{m})_{lim}$ was pH-independent second-order rate constant, and $k_{cat}$ was pH-independent turnover number. Error ranges were expressed as $\pm$ S.E.

| Enzyme     | Parameter | Equation | $(k_{cat}/K_{m})_{lim}$ or $k_{cat}$ | $pK_{a}$ | $pK_{b}$ | $pK_{c}$ |
|------------|-----------|----------|-------------------------------------|---------|---------|---------|
| Wild type  | $k_{cat}$ | Equation 1 | 5.61 | 5.43 | 5.71 |
| Y78F       | $k_{cat}$ | Equation 1 | 6.21 | 5.64 | 6.39 |
| T73A       | $k_{cat}$ | Equation 1 | 6.39 | 7.15 | 6.03 |
| Wild type  | $k_{cat}$ | Equation 2 | 3.27 | 4.0 | 5.52 |
| Y78F       | $k_{cat}$ | Equation 2 | 4.01 | 6.24 | 7.02 |
| T73A       | $k_{cat}$ | Equation 2 | 4.01 | 6.24 | 7.02 |

* The second acid dissociation constant of phosphotyrosine ($pK_{a}$) was estimated by titration curve obtained with an autotitroprocessor at 30 °C (ionic strength 0.1 M with NaCl).

**Table IV**

Kinetic parameters of wild-type and mutant VHRs obtained with various non-peptide substrates

The phosphatase activity of each mutant was measured in indicated pH in 0.1 M Tris, 0.05 M Bis-Tris, 0.05 M acetate buffer system at 30 °C. Errors were calculated by nonlinear least square fitting of the kinetic data to the Michaelis-Menten equation and expressed as ± S.D. Two duplicated sets of experiments were conducted.

| Enzyme     | Parameter | pNPP pH 6.0 | pNPP pH 7.0 | pThr pH 6.0 | pThr pH 7.0 | PMP pH 6.0 | PMP pH 7.0 |
|------------|-----------|-------------|-------------|-------------|-------------|------------|------------|
| Wild type  | $k_{cat}$ | 3.27 ± 0.10 | 2.59 ± 0.001| 1.56 ± 0.09 | 0.66 ± 0.03 | 3.61 ± 0.42| 2.31 ± 0.04|
| Y78F       | $k_{cat}$ | 0.93 ± 0.04 | 0.89 ± 0.03 | 0.74 ± 0.10 | 0.19 ± 0.005| 0.53 ± 0.01| 0.60 ± 0.03|
| T73A       | $k_{cat}$ | 0.37 ± 0.02 | 0.28 ± 0.01 | 0.53 ± 0.04 | 0.18 ± 0.03 | 0.68 ± 0.11| 0.77 ± 0.10|

The VHR activity was measured at 30 °C in 0.1 M Tris, 0.05 M Bis-Tris, and 0.05 acetate buffer system. ○, wild; ●, Y78F; ■, T73A.
Evaluation of Conserved Histidine in VHR

DISCUSSION

Low $pK_a$ values of active-site cysteines observed in various PTPs support the notion that $pK_a$ is a key determinant for thiol reactivity of the P-loop at physiological pH (31). The low $pK_a$ value (5.52 (5.44 - 5.62)) of VHR cysteine observed here is in the same range as PTP1 (5.57 ± 0.12) (32). For some PTPs like Yersinia PTP and bovine low molecular PTP, the $pK_a$ values are even lower than 5 (17, 25). Therefore, the increased $pK_a$ values found in His-123 mutants of VHR (H123A and H123F), which are at least 1.3 pH units higher than that of wild-type VHR (Table I), implicate an impairment of the normal thiol reactivity of Cys-124 by perturbing its configuration in the P-loop. The elimination of the hydrogen bond between the carbonyl oxygen of active-site Cys-124 and the imidazole ring of conserved His-123 apparently affects the cysteine at the center and at neighboring residues (4, 6, 7, 18). In contrast to the residue responsible for $pK_a$ of the cysteine itself (Fig. 1B), the thiol nucleophilicity of the active-site cysteine is not significantly affected by the mutation, although the mutation markedly diminished the $k_{cat}$ value of the cysteine would alter the catalytic capability of the enzyme. As suspected, substantial reductions of steady-state kinetic parameters were observed for mutants with pTyr as a substrate (Table II). Particularly, the 4-fold reduction in $k_{cat}$ and 11-fold reduction in $k_{cat}/K_m$ for Y78F and 14-fold reduction in $k_{cat}$ and 36-fold reduction in $k_{cat}/K_m$ for T73A are comparable with those of H123A and H123F mutants, suggesting that mutations in neighboring residues affect the Cys-124 position similarly to those in His-123. This similarity of kinetic parameters supports again that Tyr-78 and Thr-73 constitute an essential part of the histidine hydrogen bonding array and that these residues function by structurally tuning the position of His-123 in the histidine array.

A dislocation of the active-site cysteine in the P-loop means alteration of the relative position of other key residues, mainly Asp-92 and Ser-131, within the P-loop, besides the increased $pK_a$ of the cysteine itself (Fig. 1B). Therefore, mutational effects on the catalytic patterns such as the pH dependence of kinetic parameters and the effect of ethylene glycol on turnover rate ($k_{cat}$) were investigated. As evidenced by the burst kinetic traces of p-nitrophenol derived from pNPP substrate (Fig. 4), the breakdown of phosphoenzyme intermediate ($k_3$ step) is the rate-limiting step for both wild-type and Y78F mutant VHRs. This observation suggests that the overall catalytic mechanism of VHR is not significantly affected by the mutation, although the mutation markedly diminished the $k_{cat}$ value. The $k_2$ values that were virtually unchanged by the mutation implicate that the thiol nucleophilicity of the active-site cysteine is not significantly altered by the mutation in Tyr-78. A small but significant shift in pH optima of $k_{cat}/K_m$ versus pH curves observed in mutants (Fig. 5) appears to be a manifestation of subtle alteration of the Cys-124 position in the core of the P-loop. In the view of catalytic mechanism depicted in Scheme 1, $pK_a$ and $pK_c$'s ionization of constants observed from $k_{cat}/K_m$-pH profile for unprotonated species, have to be assigned to the reactive diion of pTyr substrate ($pK_a$ of pTyr) and to the nucleophilic thiolate anion ($pK_c$ of Cys-124), respectively (Table III). The residue responsible for $pK_a$ which must be protonated for activity, should be the Asp-92. The protonated aspartic acid has been known to act as a general acid in the release of leaving group ($k_2$ step). When Asp-92 was completely eliminated as in the D92N mutant, the alkaline wing (descending part) of the $k_{cat}/K_m$-$pK_c$-pH profile was totally absent (20).

Similarly, the alterations of the $k_{cat}$ values versus pH curves...
observed in the mutants imply a perturbation of the relative position of both Asp-92 and Ser-131 residues against the Cys-124. The increased $pK_a$ in hcat appears to correspond to the ascending slope of unprotonated Asp-92 (Table III). It should be kept in mind, however, that the catalytic contribution of Asp-92 in $k_{cat}$ is different from that in $k_{cat}/K_m$, since $k_{cat}$ represents the breakdown of phosphoenzyme intermediate ($k_a$ step), and the aspartate acts as a general base (Scheme 1). The protonated residue responsible for $pK_a$ derived from $k_{cat}$-$pK_a$ profile ought to be the Ser-131 residue. This assignment appears unrealistic because of the low $pK_a$ (7.02) observed in wild-type VHR. However, when considering the transient nature of the hydrolysis bond rendered by Ser-131 to thiolate to expel phosphate anion during the breakdown of phosphoenzyme intermediate and the insensitivity of the descending part of mutants to pH (absence of the $pK_a$ component), the protonated state of Ser-131 appears to be very sensitive on the neighboring hydrogen bonding network surrounding this residue. It is most likely that the hydroxyl group of Ser-131 involved in the dephosphorylation step ($k_a$) requires an optimal distance as well as a precise alignment to the leaving thiolate (Scheme 1 and Fig. 1B). This interpretation is consistent with the marked decrease of turnover rate ($k_{cat}$) observed upon mutating Ser-131 to alanine in the catalytic mechanism of VHR (14). However, the exceptionally small $k_{cat}$ values observed for pThr at pH 7.0 might implicate that the pH-dependent change of rate-limiting step occurred. In this case, the $k_{cat}$-$pK_a$ profile for pThr would reflect the ionization of groups involved in phosphorus-oxygen bond cleavage ($k_a$ step), which would mean that the unprotonated group is Cys-124 and the protonated group is Asp-92.

The reduced dependence of $k_{cat}$ on ethylene glycol nucleophile observed with Y78F (Fig. 5) again implies that the mutation affects the relative position of Asp-92 and Ser-131 to Cys-124. Since Asp-92 acts as a general base to promote nucleophilic attack of a water molecule in the breakdown of the phosphoenzyme intermediate ($k_a$ step) (Scheme 1), distortion of the Asp-92 position by mutation definitely reduces the transfer reactivity of ethylene glycol. Similarly, the conserved Ser-18 and Asp-128 residues in Stp1 have been previously recognized as critical residues involved in the transferase activity of the enzyme (33).

One characteristic of the substrate specificity of VHR is that VHR prefers aromatic phosphate to alkyl phosphate (15). The kinetic data obtained with different types of nonpeptide substrates showed substantial changes from wild-type VHR (Table IV). These kinetic data confirm the key feature of substrate IV). The kinetic data obtained with different types of nonpeptide substrates showed substantial changes from wild-type VHR (Table IV). These kinetic data confirm the key feature of substrate

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Mutational and Kinetic Evaluation of Conserved His-123 in Dual Specificity Protein-tyrosine Phosphatase Vaccinia H1-related Phosphatase: PARTICIPATION OF TYR-78 AND THR-73 RESIDUES IN TUNING THE ORIENTATION OF HIS-123

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