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Functional Significance of Conserved Glycine 127 in a Human Dual-Specificity Protein Tyrosine Phosphatase

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Abstract—Using site-directed mutagenesis and steady-state kinetic measurements, the functional role of the conserved glycine 127 in a human vaccinia H1-related phosphatase (VHR) was investigated. The mutations of Gly127 to Ala and Pro resulted in a significant decrease in k_{cat}/K_m, and increase in K_i for arsenate, indicating that flexibility at the Gly127 site has a large effect on substrate binding and catalytic activity. No substantial decrease in k_{cat}/K_m and increase in K_i values were observed for G127 deletion mutant. This showed the conformational flexibility of the PTP loop also affected the enzymatic activity of VHR. Our data suggest that the flexibility of the PTP loop in VHR is probably controlled by Gly127, and that even subtle changes in the loop flexibility may interfere with substrate binding and enzymatic reaction.

Key words: VHR, site-directed mutagenesis, steady-state kinetics, PTP loop, flexibility

Protein tyrosine phosphatases (PTPases) play an important role in signal transduction, and together with protein tyrosine kinases (PTKs) controls the level of cellular protein tyrosine phosphorylation. In the light of the biochemical and structural properties, the PTPase superfamily can be classified into three major subfamilies: 1) classic PTPases; 2) dual-specificity phosphatases, and 3) low molecular weight phosphatases [1]. Although there are limited sequence similarities among the three subfamilies of phosphatases, some essential structural features are conserved, such as the PTP loop formed by the (H/V)CXXGXXR(S/T) sequence motif, namely the active-site signature motif, and the movable loop that harbors the general acid Asp residue.

The first dual-specificity phosphatase identified (VH1), capable of dephosphorylating both phosphotyrosine and phosphothreonine/serine residues, corresponded to the H1 open reading frame in vaccinia virus [2]. Human dual-specificity protein tyrosine phosphatase VHR (for vaccinia H1-related), a negative regulator of the Erk and Jnk pathways in T cells, may play a role in the aspects of T lymphocyte physiology that depend on these kinases [3]. Using site-directed mutagenesis in conjunction with a detailed kinetic analysis, the structural and functional significance of the essential amino acid residues in VHR and other PTPases was sequentially elucidated. Within the PTP-loop, a conserved cysteine is the active-site nucleophile that forms a covalent thiol-phosphate intermediate [4-8]. An invariant arginine functions to stabilize the transition state [9-11]. The essential aspartic acid functions as a general acid and general base in the reaction mechanism [12-14]. A histidine acts as a positioning lever for the active-site cysteine [15, 16]. Either a serine or a threonine assists in stabilizing the developing negative charge during intermediate hydrolysis, making thiolate a better leaving group [17-19]. Residues Glu126 and Tyr128 were shown to be important in VHR preference for phosphotyrosine dephosphorylation and its preference for bisphosphorylated substrates, and Arg158 contributes to forming a positive pocket for phosphothreonine binding and stabilizing the active site pocket [20]. However, surprisingly, it appears that studies on the function of the conserved glycine in the PTP loop have not been reported.

Here, using site-directed mutagenesis and steady-state kinetic analysis, we explored the role of Gly127 in VHR, a highly conserved residue among the classic PTPases and the dual-specificity phosphatases except the Cdc25 phosphatases. The Gly to Pro mutation mainly decreases the flexibility of the residue to the greatest extent and is expected to be maximally disruptive to short-range interaction of the glycine residue, since the active site flexibility may play an important role in enzyme catalysis [21]. The more conservative Gly to Ala mutation is used to test whether a subtle change in struc-

Abbreviations: VHR) human tyrosine phosphatase, related to vaccinia H1-phosphatase.

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ture at this site would affect substrate binding or catalytic activity. In addition, Gly127 deletion mutation is used to probe the effect of a conformational change for PTP loop on substrate binding and catalytic activity, and the G127L mutant is used to probe the effect of a spatial barrier on substrate binding. As a result, we found that the G127A, G127P, and Gly127 deletion mutants had significantly decreased catalytic activity and substrate binding affinity, suggesting that the flexibility of the PTP loop plays an important role in catalysis.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: *p*-nitrophenyl phosphate (pNPP) from Amresco (USA); TaKaRa Ex Taq from TaKaRa BIOTECH (China); restriction enzyme DpnI from Bio-Rad (USA); isopropyl-β-D-thiogalactopyranoside (IPTG) from BBI (Canada); Chelating Sepharose™ Fast Flow from Amersham Pharmacia Biotech (Sweden).

Site-directed mutagenesis. VHR gene from pT7-7-VHR was subcloned into *Nde*I and *Xho*I sites of pET-22b to create the recombinant expression vector pET-22b-VHR by PCR. Site-directed mutagenesis of VHR was performed according to the QuikChange kit method (Stratagene, USA). The following mutagenic primers were designed:

G127P, 5′-GCTCGTCCACTGCCGGGAACCTTATAGCCGCTCCCCAACGC-3′ (sense)

5′-GCTTTGGGGAGCGGCTATAAGGTTCCCGGCAGTGGACGAGC-3′ (anti-sense)

G127A, 5′-GCTCGTCCACTGCCGGGAAGCTTATAGCCGCTCCCCAACGC-3′

5′-GCGTTGGGGAGCGGCTATAAGCTTCCCGGCAGTGGACGAGC-3′

G127L, 5′-GTCCACTGCCGGGAACTTTATAGCCGCTCCCC-3′

5′-GGGGAGCGGCTATAAAGTTCCCGGCAGTGGAC-3′

G127, 5′-GCTCCGTCCACTGCCGGGAA_TATAGCCGCTCCCCAAACGC-3′

deletion 5′-GCGTTGGGGAGCGGCTATA_TTCCCGGCAGTGGACGAGC-3′

Mutations were verified by DNA sequencing. The mutant vectors were transformed into *E. coli* strain BL21(DE3) for expression.

Expression and purification of the wild-type and mutant VHR. A 30-ml overnight culture was added to 300 ml LB containing 100 μg/ml ampicillin in a 1-liter flask and cultured at 37°C. When A_{600} reached 0.6-0.8, adding IPTG to a final concentration of 1 mM induced expression. The culture was then incubated at 37°C for an additional 4 h. The cells were harvested by centrifugation at 4°C. After the cells were lysed on ice by sonication, bacterial debris was removed by centrifugation at 12,000*g* for 20 min at 4°C. The supernatant was applied to an immobilized Ni^{2+} affinity chromatography (IMAC) column (Chelating Sepharose Fast Flow, Pharmacia Biotech) equilibrated with starting buffer containing 20 mM Tris-HCl, pH 7.8, and 0.5 M NaCl. The column was then washed in two steps with the starting buffer and wash buffer (20 mM Tris-HCl, pH 7.8, 0.5 M NaCl, and 50 mM imidazole), respectively. After washing, the bound recombinant protein was eluted with elution buffer containing 20 mM Tris-HCl, pH 7.8, 0.5 M NaCl, and 1 M imidazole. The eluate was applied to a Superdex 75 gel filtration chromatography column, and the collected fraction was dialyzed first against 0.5 M NaCl and then against 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and decreasing concentrations of NaCl (0.3 M, 0.1 M, and no NaCl, respectively). The retentate was subjected to sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE), and the homogeneity of the proteins was confirmed. The purified recombinant proteins were concentrated by ultrafiltration with Centricon Plus-20 Centrifugal Filter (Biomax membrane with 5000 NMWL, Millipore, USA) and quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, USA).

Enzyme assay and kinetics. Activity assay was performed at 30°C. The absorbance at 405 nm was measured. A three-component buffer system containing 0.1 M acetate, 0.05 M Tris, and 0.05 M Bis-Tris at pH 6.0 was used in all assays [12]. The initial velocities were measured at eight different substrate concentrations between 0.1 and 8 K_m. Kinetic parameters were determined from a linear fit of the $1/v$ versus $1/[S]$ data to the Michaelis–Menten equation using the program OriginPro 6.1. Arsenate inhibition studies were conducted at pH 6.0 and 30°C [8].

Circular dichroism spectroscopy. CD measurements were made at 25°C using a Jasco J-715 spectropolarimeter. Spectra of the protein between 195 and 250 nm were taken in a 1-mm-pathlength cell. Sample concentration was 0.2 mg/ml.

RESULTS

Purification and physical characterization. For convenience of purification with the immobilized metal ion affinity chromatography (IMAC), the recombinant VHR

was designed to have a sequence Leu-Glu-$(His)_6$ C-terminal to amino acids 2-185 of VHR. The recombinant VHR had a size of 21.4 kD (192 residues) as determined by SDS-PAGE and a molecular weight of 21,411.0 daltons by electrospray ionization mass spectrometry, a value which coincides with a calculated molecular weight of 21,411.25 daltons. The G127L mutant was expressed as an inclusion body. All proteins isolated from *E. coli* cells were purified to electrophoretic homogeneity (Fig. 1). The G127A and G127P mutants had the chromatographic properties and CD spectrum identical to that of the wild-type enzyme, indicating that their secondary structure was not significantly changed. However, the CD spectrum of the G127 deletion mutant displayed a relatively significant difference from that of the wild-type VHR, suggesting that the secondary structure of this mutant may be changed to some extent (Fig. 2).

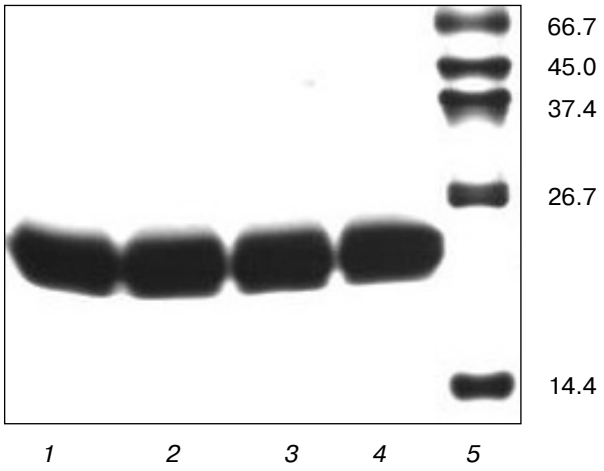

Fig. 1. SDS-PAGE of purified VHR and its mutants: *1*) VHR; *2*) G127A mutant; *3*) G127P mutant; *4*) G127 deletion mutant; *5*) molecular weight markers (kD).

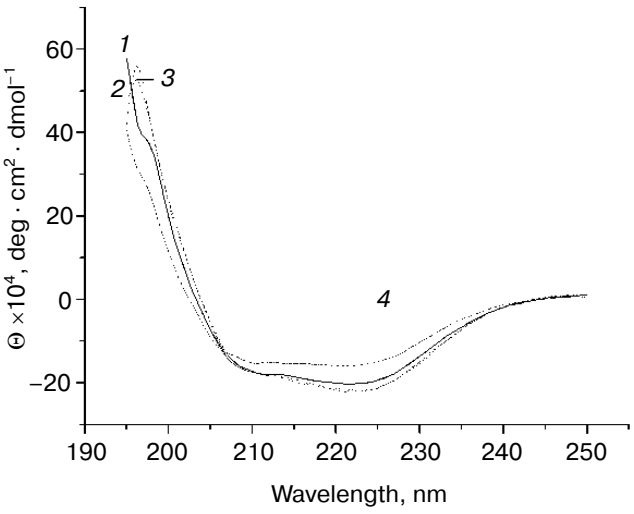

Fig. 2. Circular dichroism spectra of VHR (*1*) and its mutants: G127P (*2*); G127A (*3*); G127 deletion mutant (*4*). The spectra were recorded in three-component buffer: 0.1 M acetate, 0.05 M Tris, 0.05 M Bis-Tris, pH 6.0.

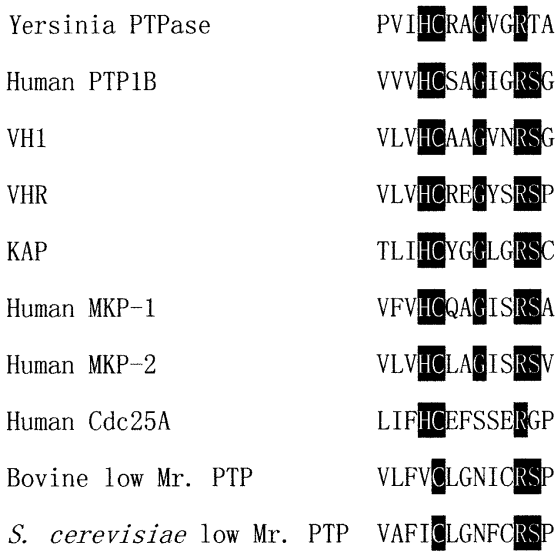

Fig. 3. PTPase signature motif sequence alignment. Highly conserved residues are highlighted.

Comparison of steady-state kinetic parameters and arsenate binding affinity for the wild-type and mutant VHR. A summary of kinetic parameters and arsenate inhibition constant is given in the table. The results showed that the histidine-tagged fusion VHR had similar steady-state kinetic parameters and arsenate inhibition constant as VHR [8, 20], suggesting that the histidine-tag did not affect significantly the biochemical properties of VHR. The Gly to Ala or Pro mutations at residue 127 of VHR resulted in a 15.4- and 167-fold reduction in k_{cat}, respectively, compared to the wild-type enzyme. The G127A and G127P mutations increased K_m value by approximately 29- and 2-fold and decreased the arsenate binding affinity by 38- and 20-fold, respectively. These results suggested that the mutation at conserved glycine 127 (Fig. 3) influenced both substrate binding and catalytic efficiency of the enzyme. The G127 deletion mutation decreased k_{cat}/K_m value by about 113-fold and K_i value by 33-fold.

Kinetic parameters and arsenate inhibition constants for wild-type and mutant VHR

VHR	k_{cat}, sec^{-1}	K_m, mM	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	K_i, mM
Wild-type	4.5 ± 0.5	1.38 ± 0.05	3200	0.019 ± 0.001
G127A	0.29 ± 0.04	40.2 ± 0.6	7.2	0.73 ± 0.03
G127P	0.0266 ± 0.0007	3.0 ± 0.1	8.9	0.38 ± 0.02
G127 deletion	0.63 ± 0.06	22.2 ± 0.9	28	0.63 ± 0.02

DISCUSSION

The only invariant residues in the PTPase superfamily are the catalytically essential Cys and Arg residues, separated by five other amino acid residues. In the PTPase signature motif (H/V)CXXGXXR(S/T), glycine is a highly conserved amino acid residue except for Cdc25A and low molecular weight phosphatases (Fig. 3). The corresponding residue, serine 433, of Cdc25A is proposed to stabilize the ionized form of the nucleophilic cysteine during catalysis [22], and a replacement of the five amino acids between the invariant active site cysteine and arginine (Glu-Phe-Ser-Ser-Glu) with Ser-Ala-Gly-Val-Gly completely abolishes activity [6]. The corresponding conserved Asn15 in the low molecular weight phosphatase makes hydrogen bonds with three other conserved residues, Ser19, Ser43, and His72, that apparently help to stabilize its left-handed conformation [23, 24]. However, Gly218 in PTP1B was proposed to play a role in phosphotyrosine binding [25]. This information shows that the serine in the Cdc25 and asparagine in the low molecular weight phosphatase may play different roles from the conserved glycine in the classic PTPase and dual-specificity PTPase.

The core active site structure elements common to the classic PTPases, the dual-specificity phosphatases, and low molecular weight phosphatases are the PTP loop and the adjacent movable loop that contains the essential Asp residue. Structure superpositions of the ligand-free and ligand-bound classic PTPase indicated that the only prominent conformational difference resides in the movable loop, and Zhang [1] suggested that this is also valid for the dual-specificity and low molecular weight phosphatases. As shown by our experiments, substrate binding may not cause a significant conformational change of the PTP loop in VHR.

According to our work, the catalytic activity of the G127A and G127P mutants was decreased by about 400-fold, and K_i value was increased by 38- and 19-fold compared with the wild-type enzyme. It is well known that proline is the least flexible residue and glycine is the most flexible residue, but the K_i value of G127A was larger than that of the G127P mutant. The reason may be that the five-member ring of the proline residue forms hydrophobic interaction with the phenyl ring of pNPP to promote substrate recognition. As deduced by Kolmodin et al. [26] from a molecular dynamics simulation, two phenyl rings of pNPP and Phe432 residue in Cdc25A stack with each other. Together, the above results indicate that the flexibility of Gly127 is mandatory for the maximal expression of VHR activity, and likely to render the PTP loop flexible. In other words, we assume that there is an induced fit process during substrate recognition by VHR. As a consequence, changing Gly127 into a different residue made the PTP loop too rigid for the VHR mutant to fit the structure of the substrate. In addition, a Gly127 deletion mutation resulted in a markedly decreased k_{cat}/K_m value and increased K_i value, and CD data showed that this mutation also caused a secondary structure change of the enzyme, suggesting that the conformational change of PTP loop must be subtle in order to maintain maximal enzymatic activity. To sum up, glycine 127, the most flexible amino acid residue, is indispensable for maximal expression of substrate binding affinity and catalytic activity. Furthermore, this functional significance can probably be assigned to the highly conserved glycine residues in the classic PTPases and dual-specificity phosphatases.

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