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Relation Between the Flexibility of the WPD Loop and the Activity of the Catalytic Domain of Protein Tyrosine Phosphatase SHP-1

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Abstract The conserved WPD loop of protein tyrosine phosphatases play an important role in the catalytic activity and the invariant aspartate residue acts as a general acid/base catalyst in the dephosphorylation reaction. In our previous report, we have demonstrated that the catalytic activities of the PTPs are influenced by the flexibility and stability of the WPD loop in its active ''open'' conformation [Yang et al., 1998]. Phosphatases with a more flexible WPD loop generally have higher specific activity. In this report, we modify the WPD loop of SHP-1 by alanine-scan mutation of the residues flanking the loop and measure their effects on the catalytic activity of the phosphatase. We show that the S418A, V424A, S426A, E427A, and P428A mutants increase the phosphatase activity, possibly due to the increased flexibility of the WPD loop, whereas the L417A, L417G and P425A mutants decrease its phosphatase activity. In addition, we propose that the two-proline residues in the WPD loop (Pro^{420} and Pro^{425} in SHP-1) work as pivotal points through a conserved hydrophobic network and allows residues between the pivotal points to have maximum flexibility in enhancing the phosphatase activity. Furthermore, our data suggest that the hydrolysis of the phosphoryl-cysteine intermediate, not its formation, is the rate-limiting step with *p*-nitrophenyl phosphate as the substrate while both the steps are rate-limiting with phosphotyrosine as the substrate. J. Cell. Biochem. 84: 47–55, 2002.

Key words: PTPs; SHP-1; catalytic domain; WPD loop; *p*-NPP; kinetics

Protein tyrosine phosphatases (PTPs) are enzymes that play an important role in controlling cell growth, differentiation, and transformation by regulating the critical phosphotyrosine levels of cellular proteins [Neel and Tonks, 1997; Zhang, 1998; den Hertog, 1999]. Precise regulation of their phosphatase activity is governed by the regulatory domains such as the SH2 domains in SHP-1 and SHP-2 [Townley et al., 1993; Zhao et al., 1993; Pei et al., 1994; Sugimoto et al., 1994; Wang and Walsh, 1997; Hof et al., 1998; Tamir et al., 2000; Zhang et al., 2000] and the substrate specific catalytic domains [Tenev et al., 1997; O'Reilly and Neel, 1998]. The specific activity of the catalytic domains of PTPs is determined by the active site, which is formed by at least three conserved amino acids: the cysteine and arginine residues in the conserved $HCX_5R(S/T)$ motif, and the aspartate residue in the flexible surface of the WPD loop [Denu and Dixon, 1998; Zhang, 1998]. The cysteine residue behaves as a nucleophile, attacking the substrate and forming the phosphoryl-cysteine intermediate. The arginine residue stabilizes the negative charge on the substrate. The aspartate residue acts as a general acid/base catalyst for the dephosphorylation reaction [Stuckey et al., 1994; Denu et al., 1995; Hengge et al., 1995; Lohse et al., 1997; Zhang, 1998]. Structural studies have shown that in order to complete the dephosphorylation reaction, the WPD loop moves approximately 6 Å from the inactive "open"

Abbreviation used: *p*-NPP, *p*-nitrophenyl phosphate.

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conformation to the active "closed" conformation and positions the aspartate residue in the active site [Stuckey et al., 1994; Jia et al., 1995; Yang et al., 1998]. Thus, the flexibility and the stability of the WPD loop in the active "closed" conformation are critical elements to control the enzymatic activity of the catalytic domains of PTPs.

The flexibility of the WPD loop is determined by the amino acids surrounding the WPD loop, which has the conserved sequence WPD(H/F)GVP for most PTPs (Fig. 1). However, for *Yersinia* PTP, which possesses the highest phosphatase activity, the highly conserved second proline residue is replaced by a valine and followed by a two-serine residue insertion (Fig. 1). In the absence of substrate, its WPD loop is observed to exchange rapidly between the "open" and the "closed" conformations with a low energy barrier between the two conformations [Wang et al., 1998]. This low energy barrier between the "open" and the "closed" conformations of the WPD loop in *Yersinia* PTP may contribute to its high specific activity. However, for other phosphatases, higher energy barriers may exist between the "open" and "closed" conformations of the WPD loop. These energy barriers would hinder the mobility of the WPD loop, which in turn will lead to a decrease in the phosphatase activity of PTPs. Therefore, mutations that increase flexibility of the WPD loop (i.e., decrease the energy barrier between the active and inactive conformations) of PTPs would also increase their specific activity.

Besides flexibility, the stability of the WPD loop in the active "close" conformation also affects the catalytic activity of the PTPs. More stable is the loop in the closed conformation, greater is the catalytic activity. This stability, however, is primarily governed by the amino acid after the aspartate residue in the WPD loop. For example, the Gln residue in *Yersinia* PTP [Stuckey et al., 1994] stabilizes the WPD loop in the enzyme substrate complex by making salt bridge with tungstate. Similarly, in PTP1B, the WPD loop is stabilized by a strong $\pi-\pi$ interaction between the conjugated rings of the Phe and Tyr residue of the substrate [Jia et al., 1995; LaMontagne et al., 1998]. Therefore, flexibility and stability of the WPD loop, which are both governed by the amino acids around it, are critical to the activities of the PTPs. Any mutation that increases the flexibility and stability of the loop would increase the activity of the PTP. To test this hypothesis, we mutated the amino acids in the WPD loop of the catalytic domain of the SHP-1 and investigated the relation between the phosphatase activity and the flexibility and stability of the WPD loop. The results of this study are described in this paper.

SHP-1	LS**WPDHGVP**--SEPG
SHP-2	RT**WPDHGVP**--SDPG
PTP1B	TT**WPDFGVP**--ESPA
PTPα	TS**WPDFGVP**--FTPI
PTPβ	TV**WPDHGVP**--ETTQ
PTPγ	TQ**WPDMGVP**--EYAL
PTPδ	TA**WPDHGVP**--EHPT
PTPκ	TG**WPDHGVP**--YHAT
PTPμ	TG**WPDHGVP**--YHAT
PTPζ	TQ**WPDMGVP**--EYSL
PTP-G1	VN**WPDHDVP**--SSFD
PTP-H1	VA**WPDHGIP**--DDSS
PTP-pez	TD**WPDHGCP**--EDVQ
YPTP	GN**WPD**QTAVSSEVTK

Fig. 1. Sequence alignment of fourteen PTPs around the WPD loop region. The identical sequences are highlighted in bold.

METHODS

Protein Expression and Purification

The wild-type and mutant catalytic domains of protein tyrosine phosphatase SHP-1 were cloned, expressed, and purified as described previously [Liang et al., 1997]. (A list of the mutants used in the study is shown in Fig. 2A). Both wild-type and mutated enzymes were purified to homogeneity as determined by appearance of a single band in SDS-PAGE. The pure fractions were pooled, buffer-exchanged to 10 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA, and concentrated to $\approx$1 mg/ml. The concentrated enzymes were stored at either 4°C for immediate use or at −20°C for future use.

Steady-State Kinetics

The kinetic measurements were performed at pH 5.0 and room temperature with the freshly purified enzymes using the published protocols under initial rate conditions [Niu et al., 1999]. The total reaction volume was 100 μl, containing both the enzyme and substrate in the working buffer (50 mM NaOAc–HOAc, pH 5.0, 2 mM EDTA, 2 mM DTT, 40% glycerol). With *p*-NPP as the substrate, the reaction was quenched by adding 900 μl of 0.2 M NaOH. Concentration of the released product, *p*-nitrophenol, was measured at A_{410} nm with an

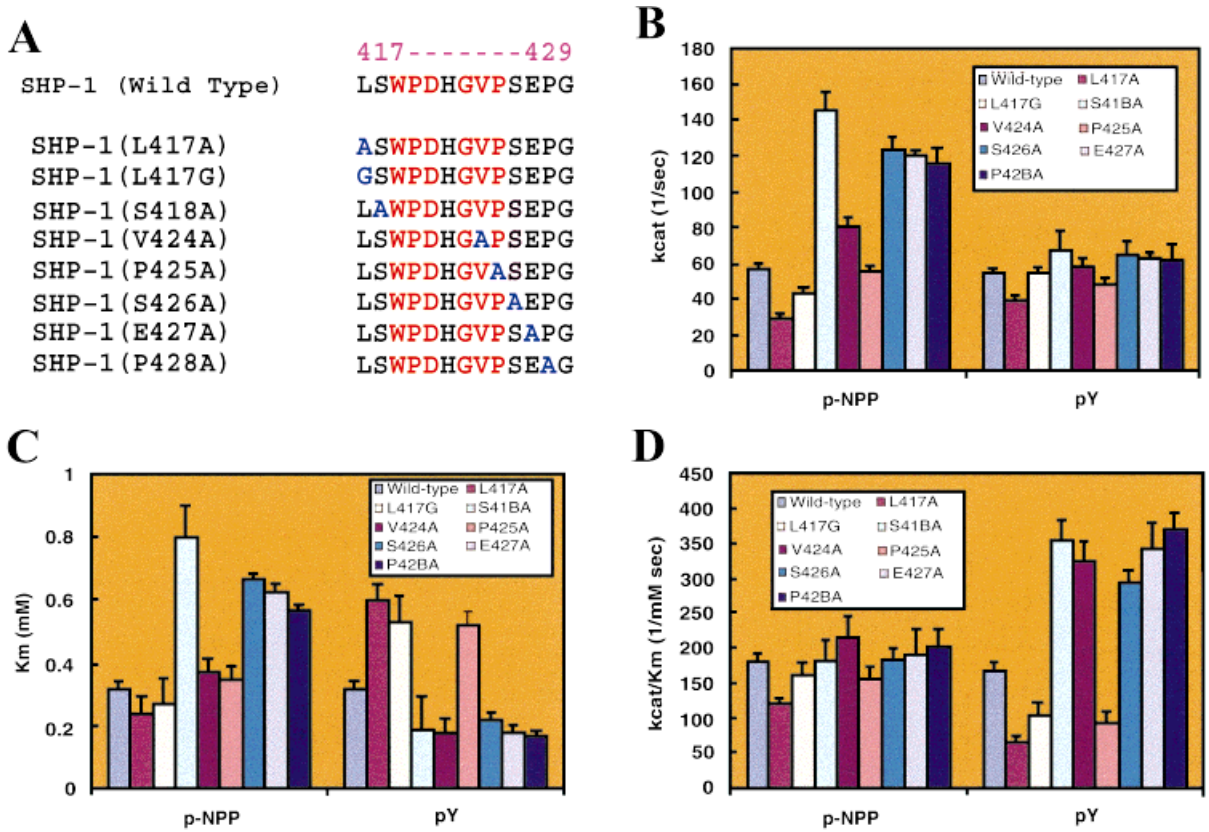

Fig. 2. **A:** A list of the alanine-scan mutants of the WPD loop used in the present study. The conserved residues in the WPD loop are labeled in red; the mutation sites are labeled in blue. **B–D:** The kinetic parameters (K_{cat}, K_m, and K_{cat}/K_m) of the wild-type and the mutated catalytic domain of SHP-1 against substrates *p*-NPP phosphotyrosine.

extinction coefficient of 17.8 mM^{-1} cm^{-1}. With the phosphotyrosine as substrate, the malachite green method [Harder et al., 1994] was used. The amount of the released inorganic phosphate was estimated from the PO_4^{3-} concentration versus A_{660} standard curve. The experimental procedure was the same as used for *p*-NPP, except that malachite green mix (3 volumes of 0.045% malachite green hydrochloride in water and 1 volume of 4.2% ammonium molybdate in 4 M HCl) was used to quench the reaction. The kinetic parameters were determined from the non-linear least-square fitting of the experimental data with the Michaelis–Menton equation.

RESULTS AND DISCUSSION

Alanine-Scan of the WPD Loop of the Catalytic Domain of SHP-1

The WPD loop has the conserved sequence WPDXGVP (X is mainly H, F or Q,) [Yang et al., 1998] in most PTPs (Fig. 1). Mutational studies around the WPD loop region has revealed the importance of these conserved amino acids [Wang et al., 1998; Yang et al., 1998; Keng et al., 1999]. For example, mutating the conserved residue tryptophane to an alanine in *Yersinia* PTP led to a decrease in its phosphatase activity [Keng et al., 1999]. We also demonstrated the significance of the corresponding amino acid His^{422} of the WPD loop in SHP-1 [Yang et al., 1998]. However, function of other amino acids in the WPD loop is still unclear. Among the residues around the WPD loop, the first proline residue (Pro^{420} in SHP-1) is highly conserved in all the PTPs, even in *Yersinia* PTP and its mutation would, therefore, be expected to disrupt the PTPs activity. That is why we did not mutate this residue in our study.

To evaluate the influence of the flexibility of the WPD loop on the phosphatase activity, we generated alanine-scan mutants of the WPD loop of the catalytic domain of SHP-1, and

TABLE I. Comparison of the Steady-State Kinetic Parameters of Wild Type and Mutants of Catalytic Domain of SHP-1 Towards Substrates *p*-NPP and Phosphotyrosine

	p-NPP*			Phosphotyrosine*		
	K_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild-type	57.6 ± 1.5	0.32 ± 0.02	182.2 ± 10.2	55.0 ± 1.2	0.32 ± 0.02	170.0 ± 9.5
L417A	29.5 ± 0.8	0.24 ± 0.01	120.8 ± 6.9	39.9 ± 1.6	0.60 ± 0.05	66.7 ± 5.1
L417G	43.9 ± 1.0	0.27 ± 0.01	162.9 ± 8.2	55.0 ± 4.1	0.53 ± 0.08	104.8 ± 16.3
S418A	145.8 ± 11.4	0.80 ± 0.10	183.1 ± 23.0	67.8 ± 1.6	0.19 ± 0.02	355.5 ± 27.9
V424A	81.3 ± 4.0	0.37 ± 0.04	217.3 ± 22.7	58.5 ± 1.4	0.18 ± 0.02	325.8 ± 27.8
P425A	55.5 ± 2.7	0.35 ± 0.04	157.8 ± 16.2	48.8 ± 1.3	0.52 ± 0.04	93.3 ± 5.2
S426A	124.1 ± 6.2	0.67 ± 0.06	184.7 ± 15.9	65.3 ± 0.7	0.22 ± 0.01	296.0 ± 9.0
E427A	120.7 ± 0.6	0.63 ± 0.07	191.9 ± 20.1	63.4 ± 1.9	0.18 ± 0.02	344.4 ± 35.0
P428A	116.6 ± 7.4	0.57 ± 0.07	203.1 ± 23.3	62.7 ± 0.8	0.17 ± 0.01	370.7 ± 15.2

*Values are expressed as mean ± SE.

measured their effects on the enzyme's specific activity (Fig. 2, Table I). These mutants were expected to modify the WPD loop, via changing the stability of the aspartate residue in the active site, and subsequently modifying the phosphatase activity of the catalytic domain of SHP-1. The phosphatase activity was measured against both *p*-NPP and phosphotyrosine at room temperature under the steady-state kinetics conditions. The kinetic parameters (k_{cat}, K_m, and k_{cat}/k_m) were obtained from the non-linear least-squares fitting of the experimental data by the Michaelis–Menton equation (Fig. 2, Table I).

The alanine-mutants of the WPD loop of the catalytic domain of SHP-1 exhibited different catalytic behaviors toward substrate, *p*-NPP, and phosphotyrosine. The substrate selectivity (k_{cat}/K_m) toward *p*-NPP was not changed for most of the mutants, relative to the wild type except for the L417A mutant where the activity was found to decrease by 30%. The substrate turnover constant (k_{cat}) against *p*–NPP was slightly decreased for the L417A and L417G mutants, almost unchanged for the P425A mutant, and slightly increased for the V424A mutant, whereas the Michaelis constant K_m was not altered (Fig. 2B and 2D, Table I). However, for the S418A, S426A, E427A, and P428A mutants, both k_{cat} and K_m were doubled, with the result there was no change in (k_{cat}/K_m). Altogether, while using *p*-NPP as the substrate, the alanine-scan mutants of the catalytic domain of SHP-1 either did not affect k_{cat} and K_m or increased them simultaneously. The (k_{cat}/K_m) value of catalytic domain of SHP-1, therefore, was nearly unchanged for the WPD loop mutants tested.

When using phosphotyrosine as the substrate, k_{cat} was almost the same for all the mutants, except for a slight decrease for the L417A mutant. However, K_ms of the L417A, L417G, and P425A mutants were almost doubled. The (k_{cat}/K_m) value of these mutants, was found to decrease by approximately 50% compared to the wild-type protein. In contrast, K_ms of the S418A, V424A, S426A, E427A, and P428A mutants were found to decrease by about one-fold resulting in an equivalent increase in the substrate selectivity. The alanine-scan mutants of the WPD loop showed changes in both K_m and k_{cat}/K_m with phosphotyrosine as the substrate. Taken together, our results suggest that the alanine-scan mutants of the WPD loop behaved differently toward *p*-NPP and phosphotyrosine and that the effect was more pronounced for the substrate phosphotyrosine as reflected from the (k_{cat}/K_m) values. (Fig. 2D, Table I).

Effects of the Alanine-Scan Mutants on Rate Constants

The dephosphorylation reaction of PTPs can be simplified as a three-step catalysis [Zhang et al., 1994; Denu and Dixon, 1998; Zhang, 1998].

$$E + S \overset{K_s}{\Leftrightarrow} ES \overset{k_2}{\rightarrow} \underset{P1}{EP} \overset{k_3}{\rightarrow} E + P2 \quad (1)$$

where P1 and P2 represent the organic product and inorganic phosphate, respectively. ES represents the enzyme–substrate complex and EP represents the phospho–enzyme complex. K_s represents the dissociation constant of the enzyme–substrate complex. k_2 and k_3 represent

the rate constants for the second and the third steps of the reaction. The kinetic parameters for the three-step reaction can be expressed as follows:

$$k_{\mathrm{cat}} = \frac{k_2 k_3}{k_2 + k_3};\ K_{\mathrm{m}} = \frac{K_{\mathrm{s}} k_3}{k_2 + k_3};\ \frac{k_{\mathrm{cat}}}{K_{\mathrm{m}}} = \frac{k_2}{k_s}. \quad (2)$$

Among these parameters, K_{s} is mainly determined by the substrate-binding pocket, whereas the rate constants k_2 and k_3 are determined by the flexibility of the WPD loop and the relative orientation of the active site aspartate residue in the WPD loop. From the experimentally determined kinetic parameters of the alanine-scan mutants, and from equation (2), we could estimate the effects of the mutants on the rate constants k_2 and k_3 and demonstrate the importance of each amino acid within the WPD loop on the dephosphorylation reaction.

For the substrate *p*-NPP, due to the electron-withdrawing effect of the *p*-nitro group and the resulting stability of the leaving *p*-nitrophenolate anion, it reacts quickly with the active-site cysteine thiolate anion (Cys^{455} in SHP-1) to form the phosphoryl-cysteine intermediate with the concomitant removal of *p*-nitrophenol. It suggests that for substrate *p*-NPP, the rate constant k_2 (i.e., formation of phosphoryl-cysteine intermediate) is larger than the rate constant k_3 (i.e., hydrolysis of the phosphoryl-cysteine intermediate) ($k_2 \gg k_3$). It follows that k_3 is the rate-limiting step. Under this condition, the kinetic parameters can be simplified as:

$$k_{\mathrm{cat}} = k_3;\ K_{\mathrm{m}} = \frac{K_{\mathrm{s}} k_3}{k_2};\ \frac{k_{\mathrm{cat}}}{K_{\mathrm{m}}} = \frac{k_2}{K_{\mathrm{s}}}. \quad (3)$$

As K_{s} (the dissociation constant of the enzyme–substrate complex) is mainly determined by the substrate-binding pocket, it is plausible that alanine-scan mutants would not change much the conformation of substrate binding pocket and, hence, would have little effects on K_{s}. As shown in Figure 2D, $k_{\mathrm{cat}}/K_{\mathrm{m}}$ is almost unchanged for all the mutants, suggesting that k_2/K_{s} also would be unchanged for all the mutants. As both K_{s} and k_2/K_{s} are unchanged for all the mutants, k_2 would not be changed for all the mutants when using *p*-NPP as the substrate. This could be due to the fast release of the stable *p*-nitrophenolate anion. Therefore, the aspartate residue in the WPD loop is not required for the second step of the reaction. Thus, the rate constant k_2 would not be affected by the mutation around the WPD loop.

Equation (3) also shows that k_{cat} and K_{m} both are proportional to k_3. The effects of the mutants on the rate constant k_3, therefore, could be determined from their effects on k_{cat} and K_{m}. Figure 2 and Table I show that k_{cat} and K_{m} were the same for the L417A, L417G, P425A, and V424A mutants as the wild type enzyme and were almost doubled for the S418A, S426A, E427A, and P428A mutants. These data suggest that the S418A, S426A, E427A, and P428A mutants increase the flexibility of the WPD loop, and therefore, increase the rate constant k_3.

Unlike *p*-NPP, in the case of phosphotyrosine, the $^{+}H_3NCHCOO^{-}$ group has an electron-donating instead of an electron-withdrawing effect. Under this condition, the formation of the phosphoryl–cysteine intermediate is no longer faster than the hydrolysis of the phosphate group from the intermediate. Hence, both the rate constants, for the second (k_2) and the third (k_3) steps of the dephosphorylation reaction are the rate-limiting steps. In other words, the rate constant k_2 is approximately equal to the rate constant k_3. The relationship between the kinetic parameters and the intrinsic rate constants can be represented by equation (2). The effects of mutation on the rate constant k_2, therefore, can be demonstrated from their effects on the substrate selectivity ($k_{\mathrm{cat}}/K_{\mathrm{m}}$). While using phosphotyrosine as the substrate, the S418A, V424A, S426A, E427A, and P428A mutants had almost doubled their substrate selectivity ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) (Fig. 2, Table I) and hence, k_2/K_{s} would also be doubled. Because k_{s} is unchanged for all the mutants, those mutants, therefore, would have doubled the rate constant k_2. This suggests that the S418A, V424A, S426A, E427A, and P428A mutants have increased the flexibility of the WPD loop, therefore resulting in an increase in k_2 value with respect to the wild type protein. However, the L417A, L417G, and P425A mutants had only 50% of their substrate selectivity values compared to the wild type protein (Fig. 2, Table I), indicating that these mutants decreased the rate constant k_2. This decrease in k_2 for the L417A, L417G, and P425A mutants seems likely to be arising from change in the flexibility and orientation of the WPD loop.

While using phosphotyrosine as the substrate, the effects of the mutants on the rate

constant k_3 cannot be compared directly from the kinetic parameters. However, if we assume that the effect of the mutants on the rate constant k_3 is similar for substrate *p*-NPP and for substrate phosphotyrosine, we predict that the S418A, S426A, E427A, and P428A mutants will double the rate constant k_3, whereas the L417A, L417G, and P425A mutants will decrease the rate constant k_3. Taken together, these results suggest that the S418A, S426A, E427A, and P428A mutants doubled both k_2 and k_3 compared to the wild type protein. Therefore, the k_{cat} of these mutants will be the same as the wild type protein and the K_ms of the mutants will be about 50% of the wild type protein. Similarly, the k_{cat} of the L417A, L417G, and P425A mutants will be the same as the wild type protein, and the K_ms of the mutants will be double of the wild type protein. These results have been observed experimentally.

For the detailed catalytic mechanism of protein tyrosine phosphatases, controversies had existed over which step of the reaction was the rate-limiting step [Harder et al., 1994; Zhang et al., 1995]. Recently, the rate-limiting step for the catalytic domain of SHP-1 was shown to be the hydrolysis of the inorganic phosphate group from the phosphoryl–cysteine intermediate with *p*-NPP as the substrate [Zhang, 1998]. Different kinetic behaviors of the alanine-scan mutants against substrates *p*-NPP and phosphotyrosine, as shown above, also suggest that the rate-limiting steps of the dephosphorylation reaction of the catalytic domain of SHP-1 are different for different substrates. These data also suggested that the S418A, S426A, E427A, and P428A mutants increase the flexibility of the WPD loop and result in the increased rate constants k_2 and k_3; however, the L417A, L417G, and P425A mutants may have somehow brought the changes in the orientation of the WPD loop, thereby resulting in the decreased rate constants k_2 and k_3 as reflected from the (k_{cat}/K_m) values (Fig. 2, Table I).

Structural Basis for the Function of the WPD Loop

During the dephosphorylation reaction, the WPD loop moves between the inactive "open" conformation and the active "closed" conformation. Functional loops of these enzymes are usually not moving freely, but restricted by hinge regions, making them more likely to accomplish the catalytic reactions. Our kinetic studies have suggested that mutants of the WPD loop which increase its flexibility will have increased rate constants, whereas those mutants which decrease the flexibility of the WPD loop will decrease rate constants. We therefore compared the kinetic results of these mutants to understand the importance of the amino acids within the WPD loop, and also to understand their influences on the movement of the WPD loop.

Structural comparison of the catalytic domain of SHP-1 with and without ligands showed that the WPD loop moved around two major hinge points that were formed by residues Trp^{419} and Pro^{420} on one side and residue Pro^{425} on the other side of the loop [Yang et al., 1998; Yang et al., 2000] (Fig. 3B). Only the four residues between the two hinges (Asp^{421}, His^{422}, Gly^{423}, and Val^{424}) were allowed to have maximum flexibility. The WPD loop can be divided into three microstructural regions as shown in Figure 3B: the tip region, the hinge regions (Hinge I, II) (Trp^{419} and Pro^{420}, and Pro^{425} in SHP-1), and the outside region (regions I, II, and Hinge III) [Yang et al., 1998; Yang et al., 2000]. The two hinges (I, II) are important in maintaining the appropriate orientation of aspartate residue (Asp^{421} in SHP-1) in the active site for accelerating catalysis. The amino acids in the tip region (Asp^{421} and His^{422}) are critical in determining the specific activity. Mutation of the aspartate residue to an alanine results in an inactive catalytic domain mutants, which have been used to "trap" the physiological substrates of PTPs [Flint et al., 1997; LaMontagne et al., 1998]. The residue His^{422} in SHP-1 in the tip region is also shown to be important in stabilizing the WPD loop in the "closed" conformation [Yang et al., 1998]. The conserved glycine residue (Gly^{423} in SHP-1) might be essential for the flexibility of the WPD loop in locating the catalytically essential aspartate residue in appropriate orientation in the active site. We therefore left it unchanged in the present study. The only amino acid with an unclear function in the WPD loop is the hydrophobic residue Val^{424}. In the catalytic domain of SHP-1, residue Val^{424} interacts with a hydrophobic pocket formed by residues Trp^{419}, Pro^{425}, Gln^{506}, and Phe^{509} (Fig. 4). These interactions would stabilize the WPD loop in the "open" conformation. Abolishing these interactions by mutating Val^{424} to an

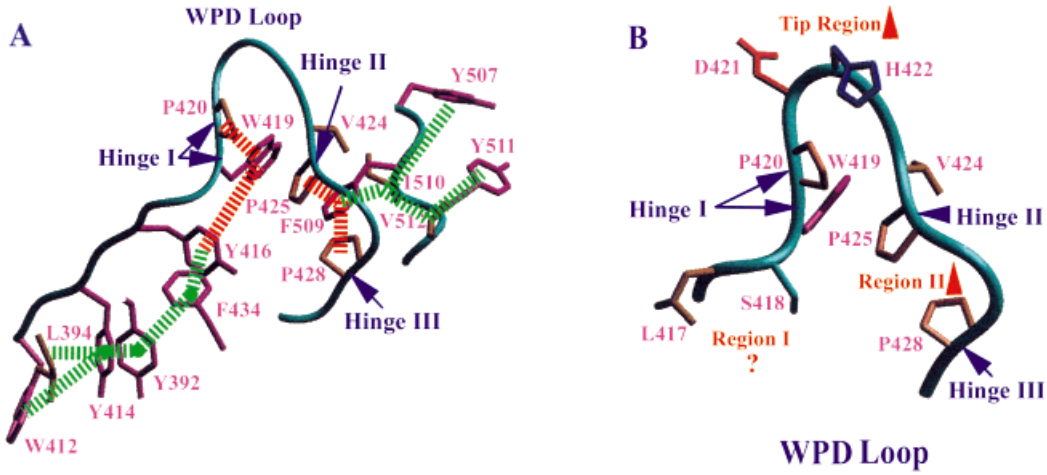

Fig. 3. **A:** The hydrophobic interactions that stabilize the three hinge regions in the WPD loop of SHP-1. Hinge I is formed by residues Trp^{419} and Pro^{420}. Hinge II is formed by residue Pro^{425}. Hinge III is formed by residue Pro^{428}. Direct interactions with the hinge residues are shown as red-dashed lines, and secondary hydrophobic interactions that stabilize the residues that are in direct contact with the hinge residues are shown in green-dashed lines. **B:** The WPD loop of SHP-1 can be divided into three regions [tip region, hinge region (hinges I, II) and outside region (regions I, II, and hinge III)].

alanine residue should increase the mobility of the WPD loop, and therefore increase the phosphatase activity of SHP-1. This has been supported by the kinetic data shown in Figure 2 and Table I.

As shown in Figure 3A, the two hinge points (Pro^{420}, Trp^{419} and Pro^{425}) were stabilized by a hydrophobic network, which is extended from the interior to the surface of the molecule. The residues forming the hydrophobic network are

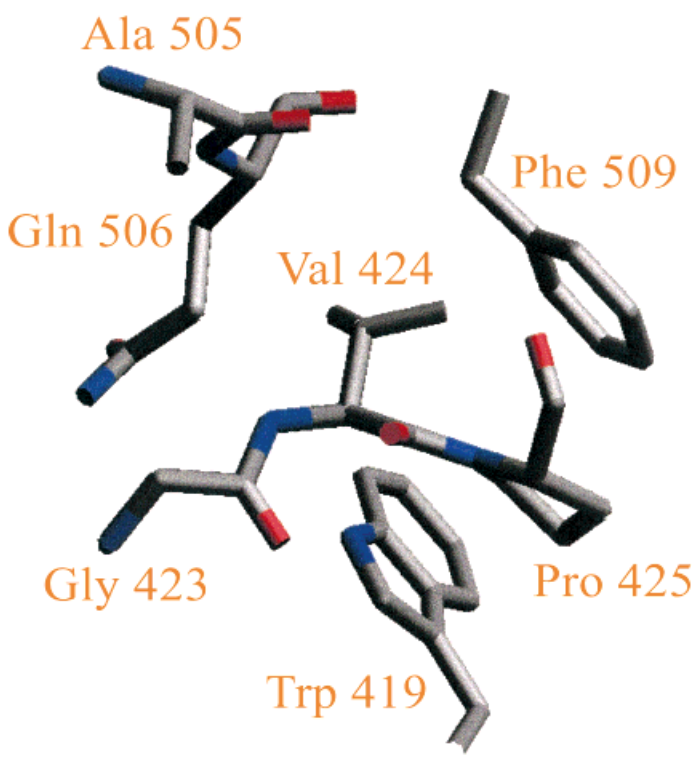

Fig. 4. A representation of the hydrophobic interactions formed between residue Val^{424} in the WPD loop and residues Trp^{419}, Pro^{425}, Gln^{506}, and Phe^{509} in the catalytic domain of SHP-1. This figure was prepared with SETOR (34).

located mainly at strands β10 and β11, the WPD loop, and helices α3 and α6. Residue Trp^{419} is immersed in a hydrophobic pocket formed by Tyr^{416}, Pro^{420}, Val^{424}, Pro^{425}, Val^{431}, Phe^{434}, Ile^{438}, Ile^{465}, Phe^{509}, and Ile^{510}, which is situated in a central position of the network (Fig. 3A). Residue Pro^{420} is stabilized by van der Waals interactions with Tyr^{416}, Trp^{419}, and Pro^{425}. Residue Pro^{425} is sandwiched with residues Pro^{428}, Val^{431}, and Phe^{509} on one side, and residues Pro^{420}, Tyr^{416}, and Trp^{419} on the other. These hydrophobic interactions maintain the structure of hinge-forming residues Trp^{419}, Pro^{420}, and Pro^{425} in the pivotal positions so that a favorable orientation for the aspartate residue is achieved. Mutations of these hinge-forming residues to alanine residues would create a hole within the hydrophobic pocket and disrupt the configuration of the WPD loop, which, in turn, would decrease the phosphatase activity. Indeed, the tryptophane to alanine mutation in *Yersinia* PTP corresponding to the W419A mutation in SHP-1 did decrease the phosphatase activity [Keng et al., 1999]. The P425A mutation also decreased k_{cat}/K_m by about one-fold in SHP-1. These results supported that Trp^{419}, Pro^{420}, and Pro^{425} behave as the major pivotal points to control the specific activity of PTPs.

Alanine-scan mutations of residues outside the two major hinges the out side region (regions I, II, and Hinge region III), including the S418A, S426A, E427A, and P428A mutants, increased the phosphatase activity against either *p*-NPP or phosphotyrosine. It appears that these mutants increased the mobility of the WPD loop while maintaining the structure of its pivotal points and relieving the constraints imposed on it by the residues from the outside region (Leu^{417}, Ser^{418}, Pro^{428}). However, the L417A and L417G mutants showed decreased catalytic activity. The reason for the decreased activity is unclear. But one possible explanation could be that residues from Region I imposed more constraints (after mutation) on the WPD loop than the residues from Region II, therefore resulting in inhibition of the mobility of the WPD loop with the net result of decrease in the phosphatase activity. Residue Leu^{417} was exposed to the solvent in the crystal structure, and formed van der Waals interactions with the side-chain hydrocarbons of Arg^{354}, Lys^{393}, and Gln^{415} [Yang et al., 1998]. Further experiments are required to demonstrate its physiological and structural importance.

CONCLUSION

Our results show that the WPD loop of SHP-1 moves around two pivotal points (Trp^{419}, Pro^{420}, and Pro^{425}), allowing maximum mobility of only the four residues (residues 421–424) between them. The two pivotal points are stabilized by a hydrophobic network that is extended from the interior to outer surface of the molecule. We also show that the rate-limiting step for the dephosphorylation reaction of the catalytic domain of SHP-1 against *p*-NPP is the hydrolysis of the inorganic phosphate from the phosphoryl-cysteine intermediate and against the phosphotyrosine both, the formation of phosphoryl-cysteine intermediate and its hydrolysis appear to be the rate limiting steps. Most important, we have generated several WPD loop mutants that are more active than the wild type catalytic domain of SHP-1. One potential application of these hyperactive phosphatases would be that they can be introduced back to the biological system to study their effects on the biological function, such as the lymphocyte development.

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