The $Cys(X)_5Arg$ Catalytic Motif in Phosphoester Hydrolysis†

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ABSTRACT: The *Yersinia* protein tyrosine phosphatase (PTPase) was identified in the genus of bacteria responsible for the plague or the Black Death and was shown to be essential for pathogenesis. The three-dimensional structure of the catalytic domain of the *Yersinia* PTPase has been solved, and this information along with a detailed kinetic analysis has led to a better understanding of the catalytic mechanism of the PTPase. Mutational and chemical modification experiments have established that an invariant Cys residue (Cys403) is directly involved in formation of a covalent phosphoenzyme intermediate. We have shown that Arg409 plays a critical role in PTPase action and that the $Cys(X)_5Arg$ active site motif forms a phosphate-binding loop which appears to represent the essential features necessary for catalysis by the PTPases, the dual specific phosphatases, and the low molecular weight acid phosphatases.

Protein tyrosine phosphorylation is a crucial event regulating numerous cellular processes such as proliferation and differentiation (Ullrich & Schlessinger, 1990; Cantley et al., 1991). The level of tyrosine phosphorylation is controlled by the opposing action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). The structure and function of PTKs have been studied extensively and only recently has attention been focused on the PTPases. More than 40 different cDNAs encoding PTPases have been cloned and characterized (Zhang & Dixon, 1994; Walton & Dixon, 1993). Amino acid sequence alignment of PTPases suggested that they contain a common evolutionary-conserved segment of approximately 250 amino acids called the PTPase catalytic domain (Zhang & Dixon, 1994; Walton & Dixon, 1993). Within this PTPase domain is a signature motif (I/V)HCXAGXGR(S/T)G (where X can be any amino acid), which is present in all PTPases. Mutational and chemical modification experiments indicate that the invariant Cys residue in this PTPase signature motif is essential for enzyme activity (Guan & Dixon, 1990; Streuli et al., 1990; Pot & Dixon, 1992; Johnson et al., 1992) and is directly involved in the formation of a covalent phosphoenzyme intermediate (Guan & Dixon, 1991; Pot et al., 1991; Cho et al., 1992).

The *Yersinia* PTPase was identified in the genus of bacteria responsible for the plague or the Black Death (Guan & Dixon, 1990). The yopH gene and its encoded phosphatase were shown to be essential for pathogenesis (Bliska et al., 1991). The *Yersinia* PTPase shows sequence identity to the mammalian PTPases within the catalytic domain (Guan & Dixon, 1990). Substitution of the conserved Cys403 residue in the PTPase signature motif by either a Ser or an Ala completely abolished both phosphatase activity and bacterial virulence (Guan & Dixon, 1990; Bliska et al., 1991). We have expressed the *Yersinia* PTPase in *Escherichia coli*, purified the protein, and obtained diffraction-quality crystals (Zhang et al., 1992). A three-dimensional structure of the catalytic domain of the *Yersinia* PTPase has been solved (Stuckey et al., 1994). In addition, Barford and his colleagues have recently reported the structure of the mammalian PTPase, PTP1B (Barford et al., 1994).

We have also undertaken a detailed kinetic analysis of the *Yersinia* PTPase in order to understand the mechanism of phosphate monoester hydrolyses by this family of enzymes (Zhang et al., 1994a). Amino acid sequence alignment of over 30 PTPases, which include bacterial, yeast, and mammalian enzymes, suggests that there are only 21 residues (including the catalytically essential Cys residue) that are invariant among all PTPases (Zhang et al., 1994b). We demonstrated that among the 21 invariant residues, His402 in the *Yersinia* PTPase has a stabilizing effect on the active site Cys403 thiolate anion (Zhang & Dixon, 1993), and Asp356 and Glu290 act as the general acid and general base in the PTPase-catalyzed reaction (Zhang et al., 1994b). In this report, we show that the invariant Arg409 residue plays a critical role in substrate recognition and transition-state stabilization. Cys403 and Arg409 constitute a $Cys(X)_5Arg$ active site motif essential for PTPase activity. This motif is not restricted to the PTPases, and it appears to form a phosphate-binding loop (Stuckey et al., 1994) which contains structural elements necessary for catalysis by the PTPases, the dual specific phosphatases, and the low molecular weight acid phosphatases.

EXPERIMENTAL PROCEDURES

Materials. *p*-Nitrophenyl phosphate (*p*-NPP) was from Fluka. Mutagenesis kits were from Bio-Rad. All other chemicals were purchased from Sigma.

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Table 1: Kinetic Parameters of the *Yersinia* PTPase and Its Conserved Arg Mutants at pH 6.0

Yersinia PTPase	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	*Yersinia* PTPase	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	345 ± 5.5	2.60 ± 0.12	133				
R216A	193 ± 5.8	4.39 ± 0.32	44.0				
R228A	160 ± 6.1	3.80 ± 0.36	42.1	R228K	74.0 ± 2.1	8.51 ± 0.54	8.70
R236A	351 ± 16.9	3.53 ± 0.44	99.4				
R295A	361 ± 9.1	2.33 ± 0.16	155				
R409A	$4.23 \times 10^{-2} \pm 1.3 \times 10^{-3}$	66.3 ± 3.4	6.38×10^{-4}	R409K	$3.38 \times 10^{-2} \pm 3.8 \times 10^{-4}$	5.02 ± 0.29	6.73×10^{-3}
R437A	66.6 ± 2.8	19.3 ± 1.9	3.45	R437K	283 ± 9.3	3.61 ± 0.30	78.4
R440A	0.115 ± 0.0054	23.2 ± 0.80	4.96×10^{-3}	R440K	3.78 ± 0.06	10.7 ± 0.60	0.353

Site-Directed Mutagenesis. Alanine substitutions for R216, R228, R236, R295, R409, R437, and R440 or lysine substitutions for R228, R409, R437, and R440 of the *Yersinia* PTPase were made by site-directed mutagenesis according to the procedure of Kunkel et al. 1987). The oligonucleotide primers used for the desired substitutions were as follows: R216A, ACGAATGATCCGGCTTACTTACAAGCCTGC; R228A, GGTGAAAAGCTAAACGCATT TAGAGATATTCA; R236A, ATTCAATGCCGTGCGCAAACCGCA; R295A, TAGCCAA TCAAGCATTCGGTATGC; R409A, GGTGTTGGCGCTACTGCGCAACT; R437A, AGCCAAATGGCAGTACAAAGA; R440A, CGAGTACAAGCAAATGGTATTAT; R228K, AAGCTAAACAAATTTAGAGATAT; R409K, GCGGGTGTTGGCAAAACTGCGCAA CTGAT; R437K, AGCCAAATGAAAGTACAAAGAAA, and R440K, CGAGTACAA AAAAATGGTA. All of the mutations were verified by DNA sequencing (Sanger et al., 1977).

Expression and Purification of the Recombinant Enzymes. The wild-type *Yersinia* PTPase and all of the mutant proteins were expressed in *E. coli* and purified to homogeneity as described (Zhang et al., 1992). Substitutions at residues 228 and 440 yielded inclusion bodies. In these instances, the pellet was dissolved in 3 mL of 8 M urea in lysate buffer (100 mM acetate, 100 mM NaCl, 1 mM EDTA, pH 5.7), and then the protein was refolded by dropwise dilution into 300 mL of lysate buffer at 4 °C (Zhang et al., 1992).

Assay. All enzyme assays were performed at pH 6 and 30 °C. The buffer contained 50 mM succinate and 1 mM EDTA with the ionic strength kept at 0.15 M using NaCl. Initial rate measurements for the enzyme-catalyzed hydrolysis of aryl phosphate monoesters were conducted as previously described (Zhang et al., 1992). Kinetic parameters were determined from a direct fit of the v vs [s] data to the Michaelis–Menten equation using the nonlinear regression program Grafit (Leatherbarrow, 1990). Since a tight-binding specific PTPase inhibitor is not available at the moment, k_{cat} values are calculated using protein concentration determined by UV absorbance. Arsenate inhibition studies were conducted at pH 7 and 30 °C in 50 mM 3,3-dimethylglutarate and 1 mM EDTA with the ionic strength kept at 0.15 M using NaCl. Initial rates were measured at five different inhibitor concentrations, and k_{cat}, K_m and K_i were obtained using the program Grafit (Leatherbarrow, 1990).

Spectroscopy. UV spectra of the *Yersinia* PTPases were recorded at 25 °C using a Perkin-Elmer λ6 spectrophotometer in 100 mM acetate, 100 mM NaCl, and 1 mM EDTA, pH 5.7, buffer. CD spectra were recorded at 20 °C with a JASCO J-700 spectropolarimeter. The spectra for the wild-type and the Arg mutants of the *Yersinia* PTPase were taken at protein concentrations of approximately 0.25–0.32 mg/mL, using a 0.1-mm quartz cuvette. The following settings were used: wavelength range, 200–250 nm; bandwidth, 1 nm; time constant, 4.0 s; step resolution, 0.1 nm; scan speed, 50 mdeg/min; sensitivity, 50 mdeg/cm. Each spectrum represents an average of four scans with the baseline subtracted. All CD measurements were conducted in 50 mM acetate, 50 mM NaCl, and 0.5 mM EDTA, at pH 5.7.

RESULTS AND DISCUSSION

The *Yersinia* enzyme is the most active PTPase known (Zhang et al., 1992) and is an ideal candidate for structure–activity analysis. It contains the active site signature motif (I/V)HCXAGXGR(S/T)G (where X can be any amino acid), which is present in all PTPases. Of 21 invariant residues present in all PTPase catalytic domains in species ranging from bacteria to mammals (Zhang et al., 1994b), there are four invariant Arg residues: Arg228, Arg409, Arg437, and Arg440. The role of these four Arg residues was examined by site-directed mutagenesis. We initially produced Arg to Ala substitutions at each site. In addition, we made Arg to Lys substitutions in order to maintain the positive charge at each positions. We also produced Arg to Ala mutations at Arg216, Arg236, and Arg295, since each of these residues is either conserved in previous sequence alignments or has a positive charge at this residue in most PTPases (i.e., Arg for Lys substitution). All of the wild type as well as the mutant proteins were overexpressed in *E. coli* and purified to homogeneity as described (Zhang et al., 1992). Table 1 summarizes kinetic parameters, k_{cat}, K_m, and k_{cat}/K_m, of the wild-type and the mutant enzymes at pH 6.0, using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. The PTPase mutants R216A, R236A, and R295A had k_{cat} and k_{cat}/K_m values similar to those of the wild-type *Yersinia* PTPase, suggesting that they play minor roles in substrate binding or catalysis. The kinetic parameter k_{cat} relates to the rate-limiting step of the PTPase catalysis, whereas the term k_{cat}/K_m contains kinetic information about PTPase catalysis up to the phosphorylation step, as well as serving as the specificity constant for the substrate. Of the four invariant Arg mutants, R228A and R437A displayed kinetic parameters modestly different from those of the wild-type enzyme with a 2- and 5-fold decrease in k_{cat} and a 3.2- and 38-fold decrease in k_{cat}/K_m, respectively, while R440A exhibited a k_{cat} and k_{cat}/K_mthat is 3000- and 26 800-fold lower than those of the wild-type enzyme. We have examined the possibility that a Lys substitution at Arg228, Arg409, Arg437, and Arg440 could replace the function of an Arg residue by partially restoring the positive charge at this position in the protein. Surprisingly, R228K is an even worse catalyst than R228A, with a 5-fold decrease in k_{cat} and 15-fold decrease in k_{cat}/K_m in comparison with the wild-type enzyme. Inter-

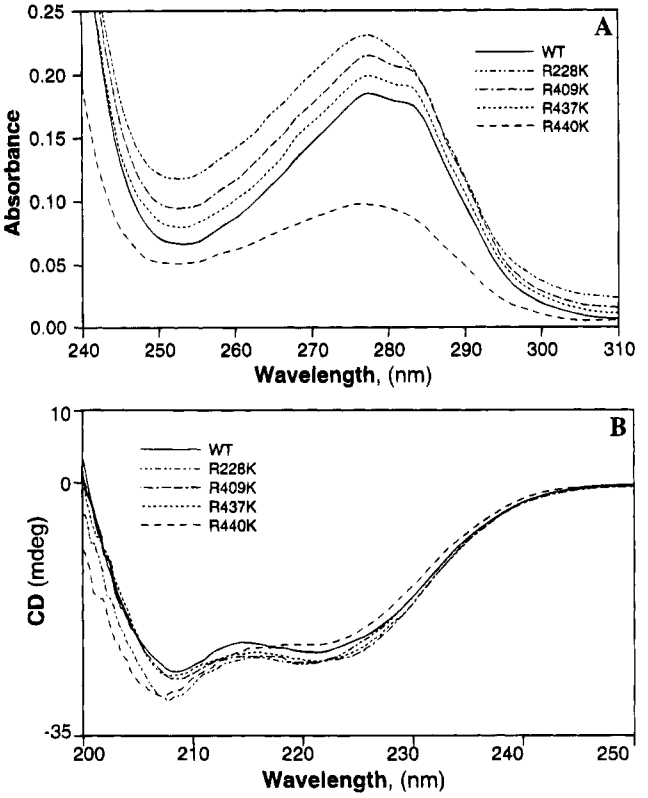

FIGURE 1: (A) Ultraviolet absorption spectra of the *Yersinia* PTPases. The protein concentration was as follow: 0.516 mg/mL for the wild-type *Yersinia* PTPase, 0.637 mg/mL for R228K, 0.598 mg/mL for R409K, 0.558 mg/mL for R437K, and 0.271 mg/mL for R440K. (B) CD spectra of the *Yersinia* PTPases. The protein concentration was as follow: 0.258 mg/mL for wild type, 0.318 mg/mL for R228K, 0.299 mg/mL for R409K, 0.279 mg/mL for R437K, and 0.250 mg/mL for R440K.

estingly, the Arg to Lys substitution at residue 437 produced a mutant PTPase that is kinetically indistinguishable from the wild-type enzyme. The Arg to Lys substitution at residue 440 generated a catalyst that was dramatically improved over mutant R440A, with a 33-fold increase in k_{cat} and a 71-fold increase in k_{cat}/K_m, respectively. Because Arg228 and Arg440 mutants were produced as inclusion bodies and had to be refolded *in vitro*, we were concerned that, after refolding, their structures may not be reflective of the native structure. All of the Arg mutants had chromatographic properties identical to that of the wild-type enzyme. Figure 1 shows the UV and CD spectra of the wild type as well as those of R228K, R409K, R437K, and R440K. Both UV and CD spectra suggested that mutant R228K and R440K had altered structures (Figure 1). In contrast, both Arg409 and Arg437 mutants had spectra indistinguishable from that of the wild-type enzyme. Similar results were obtained for the Arg to Ala mutants; i.e., R409A and R437A had spectra similar to that of the wild-type enzyme while R228A and R440A had altered structures (data not shown). It is perhaps not surprising that mutations at residue R440 cause a deleterious effect on both enzyme activity and structure. Arg440 is completely buried in the crystal structure and forms H-bonds with peptide carbonyls of residues in the phosphate-binding loop (Stuckey et al., 1994). The side chains of R228 and R437 are located at the surface of the molecule, surrounding the catalytic site, and are exposed to solvent. They are probably not important for catalysis.

Table 2: Arsenate Inhibition Constants for the *Yersinia* PTPase and Its Conserved Arg Mutants at pH 7.0

Yersinia PTPase	K_i (mM)	*Yersinia* PTPase	K_i (mM)
WT	0.882 ± 0.050		
R295A	0.952 ± 0.034		
R409A	41.1 ± 3.7	R409K	15.5 ± 0.70
R437A	3.72 ± 0.33	R437K	1.13 ± 0.045

The Arg to Ala mutation at residue 409 results in a dramatic change in both k_{cat} and k_{cat}/K_m when compared to the wild-type enzyme. Similar mutations made in the equivalent Arg residue of two receptor-like PTPases, LAR and CD45, resulted in complete loss of enzymatic activity as measured from bacterial extracts (Streuli et al., 1990; Johnson et al., 1992). It is not clear from such studies whether the Arg residue is essential for PTPase structure or catalysis, or both. The extraordinary reactivity of the *Yersinia* PTPase has made it possible to measure k_{cat} and K_m values for both the R409A and R409K site-directed mutants. We can now address the functional significance of Arg409 in terms of substrate binding and catalysis. A 8200-fold decrease in k_{cat} and a 26-fold increase in K_m were observed for the R409A mutant. Interestingly, the R409K mutant displayed a k_{cat} value similar to that of R409A; however, its apparent K_m value improved significantly and was only 1.9-fold higher than that of the wild-type enzyme. This suggests that Arg409 plays a significant role in substrate binding and an even more important role in transition-state stabilization. In order to probe the function of the invariant Arg residues on substrate binding in greater detail, we also determined the competitive inhibition constants for arsenate (Zhang & Dixon, 1993) with each mutant. Table 2 summarizes the effect of Arg substitution on arsenate binding. Residue Arg409 is the only amino acid substitution that affects the affinity of the *Yersinia* PTPase for arsenate. The R409A mutation decreases the arsenate binding affinity by 47-fold while R409K decreases the arsenate binding affinity by 18-fold. The R437A mutant shows a 4.2-fold reduction in the arsenate binding affinity, and a Lys residue can effectively substitute for Arg437 in terms of inhibitor binding. It was not possible to obtain a complete set of binding constants for tungstate because the recombinant enzyme precipitates in the presence of high concentrations of this reagent.

Tungstate is a competitive inhibitor of the *Yersinia* PTPase with a binding constant of 61 μM at pH 7.0 and ionic strength of 0.15 M. The crystal structures of both the *Yersinia* PTPase–tungstate (Stuckey et al., 1994) and the mammalian PTP1B–tungstate (Barford et al., 1994) complexes show that the tungstate oxygen atom(s) ion(s) pair with the positively charged Arg409 and Arg221, respectively. The PTP1B structure indicates a single hydrogen bond between the Nη1 of Arg221 and the tungstate oxygen atom (Barford et al., 1994). The *Yersinia* PTPase structure indicates that there are two hydrogen bonds between the guanidinium group of Arg409 and two of the tungstate oxygen atoms (Stuckey et al., 1994) (Figure 2A). Tungstate oxygens labeled a and b form hydrogen bonds with the Nϵ and Nη of Arg409. The tungstate oxygen denoted as c is hydrogen bonded to the NH amides of the peptide backbone making up the P-loop (Stuckey et al., 1994). The tungstate oxygen atom denoted as d is projecting out of the active site pocket. This oxygen

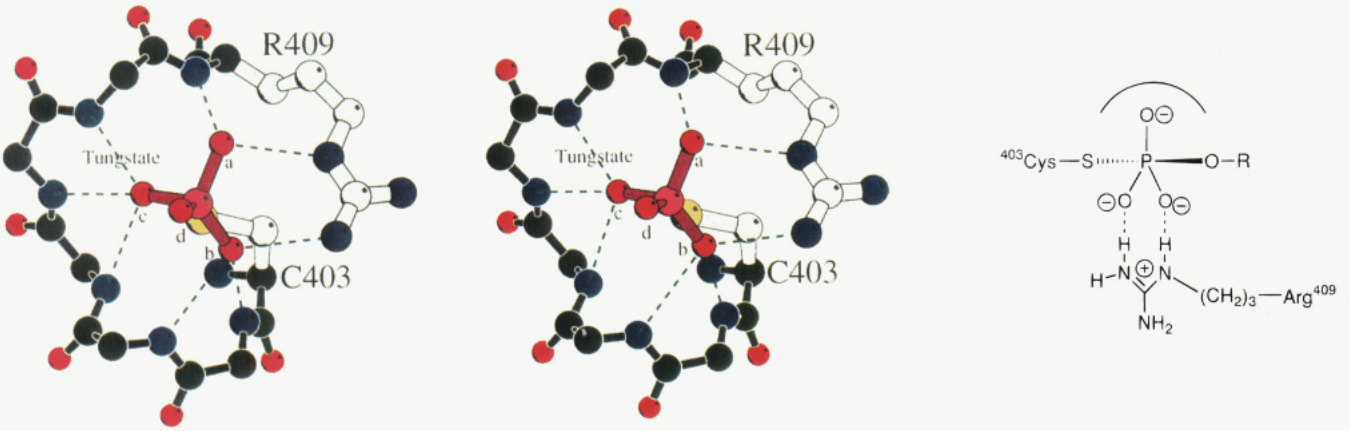

FIGURE 2: PTPase active site. (A, left) Stereoview of the phosphate-binding loop or the P-loop with WO_4^{2-} in the *Yersinia* PTPase (Stuckey et al., 1994). Only the peptide backbone from residues 403−409 is shown. Atoms are denoted as follows: nitrogens (blue), oxygens (red), main-chain carbons (black), side-chain carbons (gray), the sulfur of Cys403 (yellow), and tungstate (magenta). Hydrogen bonds are shown as green dotted lines. The figure was drawn using MOLSCRIPT (Kraulis, 1991). (B, right) Suggested pentacoordinated transition-state structure. −OR is a tyrosine-containing peptide or a hydroxyl group.

atom most likely corresponds to the oxygen atom present in the substrate scissile phosphate ester bond. The role of the invariant Cys residue in the catalytic mechanism is consistent with the attack by the thiol anion on the phosphate ester with subsequent cleavage of the P−O bond resulting in the liberation of the tyrosine-containing peptide or protein (Zhang & Dixon, 1994; Zhang et al., 1994a). Figure 2A shows that the thiol anion of Cys403 is positioned at the base of the active site and it would likely attack the phosphate ester forming a trigonal-bipyramidal transition state (see Figure 2B). The side chain of Arg409 is in an ideal position for effective stabilization of the transition state (Figure 2B). Cleavage of the phosphoester linkage would then produce the thiol−phosphate intermediate with release of −OR, the tyrosine-containing peptide. This intermediate has been trapped using ^{32}P-labeled substrate (Guan & Dixon, 1991; Pot et al., 1991), and kinetic analysis has demonstrated that it is on the reaction pathway (Cho et al., 1992). Hydrolysis of the phosphoenzyme intermediate would most likely require water to attack in the same axial position from which the tyrosine-containing leaving group departed. The attack of water on the phosphoenzyme intermediate would again form a trigonal-bipyramidal transition state, where R represents a hydrogen atom. The subsequent cleavage of the thiol−phosphate bond would regenerate the thiol anion of Cys403 and yield the other product of the reaction, inorganic phosphate.

Arg409 plays an important role in the rate-limiting step in catalysis and transition-state stabilization for the *Yersinia* PTPase (Table 1). This is reflected by the reduction in both k_{cat}/K_m and k_{cat} values for the R409A mutant. Studies have shown that a guanidinium group (present in arginine) is ideally suited for interaction with phosphate by virtue of its planar structure and its ability to form multiple hydrogen bonds with the phosphate moiety (Cotton et al., 1973). We estimate that at pH 6.0 and 30 °C, using *p*-NPP as a substrate, the Arg409 to Ala mutation leads to a loss in binding energy of 1.95 and 2.31 kcal/mol for *p*-NPP and arsenate, respectively. These binding energies are in close agreement with what would be expected when a negatively charged phosphate group is interacting with the positively charged Arg residue via either one or two hydrogen bonds (Meiering et al., 1991). As noted above, this binding energy is consistent with the X-ray crystallographic studies of the *Yersinia* PTPase and with the structure of PTP1B. The increases in the free energies of activation due to the R409A mutation for the first irreversible step of the reaction as measured by k_{cat}/K_m and for the rate-limiting step of the reaction as measured by k_{cat} are calculated to be 7.37 and 5.42 kcal/mol, respectively. The ability of the guanidinium group to form a coplanar bidentate complex with two equatorial oxygen atoms present on the phosphate during catalysis provides a plausible mechanism for stabilization of the increasing negative charge in the trigonal-bipyramidal transition state(s) (Figure 2). Consistent with this notion, the R409K mutant showed no improvement in k_{cat} when compared to R409A. The geometry associated with the amino group of the Lys side chain would not be expected to form a coplanar bidentate complex with the trigonal bipyramidal transition state as can be accommodated by the guanidinium group of an Arg residue (Figure 2B). The fact that a Lys residue at position 409 can partially replace the Arg residue in terms of substrate and inhibitor binding while at the same time it cannot substitute for the Arg in catalysis suggests that the transition state(s) likely employ(s) the unique structural properties of the guanidinium side chain of Arg409 (Figure 2).

Are the invariant Cys and Arg residues present in the PTPase signature motif, (I/V)HCXAGXGR(S/T)G, found in other related or unrelated proteins? There is a group of phosphatases that can utilize substrates containing phosphoserine/threonine, as well as phosphotyrosine. This group of enzymes, called VH1-like phosphatases or dual specific phosphatases, includes the vaccinia phosphatase VH1 (Guan et al., 1991) and the mammalian enzymes known as CL100 (Keyse & Emslie, 1992) and VHR (Ishibashi et al., 1992), as well as the cell cycle regulator p80^{cdc25} (Millar et al., 1991; Sadhu et al., 1990). The dual specific phosphatases also appear to utilize a thiol phosphate intermediate in phosphate ester hydrolysis (Zhou et al., 1994). Although the dual specific phosphatases display amino acid sequence identity to the PTPases within the active site, they have limited sequence identity to other PTPases outside the active site (Keyse & Emslie, 1992; Ishibashi et al., 1992; Kwak et al., 1994). The dual specific phosphatase, VH1, also has an absolute requirement for the active site Cys and Arg residue for enzymatic activity (Guan et al., 1991; Gautier et al., 1991). More striking, however, is another group of phos-

Phosphatase	Sequence
Yop51	P V I H C R A G V G R T A
PTP1B	P V V V H C S A G I G R S G
LAR	P M V V H C S A G V G R T G
VHR	R V L V H C R E G Y S R S P
VH1	P V L V H C A A G V N R S G
mouse cdc25	I V F L C E F S S E R G P
Low Mr. Phosphatase	V L F V C L G N I C R S P

FIGURE 3: Cys(X)$_5$Arg motif. The active site sequence alignment: Yop51 (Michiels & Cornelis, 1988), PTP1B (Chernoff et al., 1990), LAR (Pot et al., 1991), VHR (Ishibashi et al., 1992), VH1 (Guan et al., 1991), mouse cdc25 (Millar et al., 1991), and low M_r phosphatase (Wo et al., 1992).

phatases, originally termed low molecular weight acid phosphatases (Heinrikson, 1969), which also form a thiol phosphate intermediate (Zhang & Van Etten, 1991; Wo et al., 1992). In spite of the catalytic similarities between the PTPases and the low molecular weight acid phosphatases, there is no significant sequence identity between these two groups of enzymes. However, the low molecular weight acid phosphatases have two residues which appear to be essential for catalysis, namely, Cys12 and Arg18 (Cirri et al., 1993; Davis et al., 1994). Figure 3 shows a sequence comparison of the active site regions of representative PTPases, dual specific phosphatases, and a low molecular weight acid phosphatase. The only invariant residues in these three families of phosphatases are the catalytically essential Cys and Arg residues. The invariant Cys residue is always preceded by a cluster of hydrophobic residues, and the invariant Arg residue is surrounded by amino acids such as Gly, Ser, Thr, Asn, or Pro. These amino acids are required to maintain the structural integrity of the phosphate-binding loop (Stuckey et al., 1994). The structure of the phosphate-binding loop or P-loop, and the invariant Cys and Arg residues, in the *Yersinia* PTPase and PTP1B, has been conserved from bacteria to mammals (Zhang & Dixon, 1994; Zhang et al., 1994b). Remarkably, the structure of phosphate-binding loop in the low molecular weight phosphatases is similar to the corresponding region in PTP1B and the *Yersinia* PTPase (Su et al., 1994; Zhang et al., 1994).

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