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Protein-Tyrosine Phosphatases: Biological Function, Structural Characteristics, and Mechanism of Catalysis

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ABSTRACT: The protein-tyrosine phosphatases (PTPases) superfamily consists of tyrosine-specific phosphatases, dual specificity phosphatases, and the low-molecular-weight phosphatases. They are modulators of signal transduction pathways that regulate numerous cell functions. Malfunction of PTPases have been linked to a number of oncogenic and metabolic disease states, and PTPases are also employed by microbes and viruses for pathogenicity. There is little sequence similarity among the three subfamilies of phosphatases. Yet, three-dimensional structural data show that they share similar conserved structural elements, namely, the phosphate-binding loop encompassing the PTPase signature motif (H/V)**C**$(X)_5$**R**(S/T) and an essential general acid/base Asp residue on a surface loop. Biochemical experiments demonstrate that phosphatases in the PTPase superfamily utilize a common mechanism for catalysis going through a covalent thiophosphate intermediate that involves the nucleophilic Cys residue in the PTPase signature motif. The transition states for phosphoenzyme intermediate formation and hydrolysis are dissociative in nature and are similar to those of the solution phosphate monoester reactions. One strategy used by these phosphatases for transition state stabilization is to neutralize the developing negative charge in the leaving group. A conformational change that is restricted to the movement of a flexible loop occurs during the catalytic cycle of the PTPases. However, the relationship between loop dynamics and enzyme catalysis remains to be established. The nature and identity of the rate-limiting step in the PTPase catalyzed reaction requires further investigation and may be dependent on the specific experimental conditions such as temperature, pH, buffer, and substrate used. In-depth kinetic and structural analysis of a representative number of phosphatases from each group of the PTPase superfamily will most likely continue to yield insightful mechanistic information that may be applicable to the rest of the family members.

KEY WORDS: cancer, diabetes, infectious diseases, transition state, kinetic isotope effect, loop dynamics, rate-limiting step.

I. INTRODUCTION

A fundamental mechanism that a cell utilizes to control its biological processes is via protein phosphorylation and dephosphorylation. It was estimated that one-third of cellular proteins are phosphorylated. The majority of protein phosphorylation in eukaryotic cells occurs on Ser or Thr residues (Roach, 1991), and Tyr phosphorylation only accounts for 0.01 to 0.05% of the total protein phosphorylation (Hunter and Sefton, 1980). However, after oncogenic transformation (Levinson et al., 1980, Sefton et al., 1981) or growth factor stimulation (Ushiro and Cohen, 1980), the level of Tyr phosphorylation increases to 1 to 2% of the total protein phosphorylation in the cell. It was not until the early 1980s that attention has been given to protein tyrosine phosphorylation (Hunter, 1996). Although Tyr phosphorylation occurs to a much smaller extent, it has become clear that Tyr phosphorylation is involved in the regulation of numerous cell functions, including passage through the cell cycle, proliferation and differentiation, metabolism, motility, cytoskeletal organization, neuronal development, cell-cell interactions, gene transcription, and the immune response (Hunter, 1995 and 1996; Tonks and Neel, 1996).

In vivo, the level of tyrosine phosphorylation in a given protein is regulated by the opposing actions of protein-tyrosine kinases (PTKs, EC 2.7.1.112) and protein-tyrosine

phosphatases (PTPases, EC 3.1.3.48). PTKs are enzymes that catalyze the transfer of the γ-phosphoryl group of ATP to the 4-hydroxyl of tyrosyl residues within specific protein/peptide substrates. PTPases are hydrolytic enzymes that remove the phosphoryl group from the phosphorylated tyrosine residue(s). The protein kinase family is extraordinarily large and a recent estimate suggested that as many as 2000 protein kinases genes, with up to 50% being PTK genes, may be encoded within the human genome (Hunter, 1996). As PTKs have been identified as growth factor receptors, protooncogenes, and cell cycle regulators, extensive structural and functional studies have been focused on PTKs (Hunter, 1987; Hunter, 1991). It was originally thought that PTKs were the key enzymes controlling the tyrosine phosphorylation *in vivo*, and that PTPases were small in number and functioned essentially as "house-keeping" enzymes to constitutively reverse the action of PTKs. This view has since been proven to be incorrect. Since the isolation and characterization of the first PTPase, PTP1B (Tonks et al., 1988a,b), cDNA cloning using polymerase chain reaction and low-stringency hybridization have led to the identification of more than 100 PTPases. In fact, it turned out that protein phosphatases are also comprised of a large family of enzymes that parallel protein kinases in terms of structural diversity and complexity. Based on genomic analysis, it was predicted that humans could have as many as 1000 protein phosphatase genes (Hunter, 1995), with up to 500 being PTPase genes (Tonks and Neel, 1996). The exact functional roles of PTPases in cellular processes regulated by Tyr phosphorylation are currently under intensive investigation in numerous laboratories. Papers published over the last several years document crucial physiological roles for PTPases in a variety of mammalian tissues and cells (Charbonneau and Tonks, 1992; Walton and Dixon, 1993; Tonks and Neel, 1996; and Neel and Tonks, 1997). Because tyrosine phosphorylation of proteins is reversible and dynamic *in vivo* and the phosphorylation states of proteins are governed by the opposing actions of PTKs and PTPases, it becomes clear that PTKs and PTPases play equally important roles in signal transduction in eukaryotic cells. Thus, comprehension of physiological roles of protein tyrosine phosphorylation, and its potential as a mechanism for reversible modulation of protein function and cell physiology, must necessarily encompass the characterization of PTPases in addition to the PTKs. The goal of this review is to discuss how current genetic, structural, biophysical, and enzymological approaches have been combined to provide a better understanding of the structure and function relationship of PTPases.

II. CLASSIFICATION

Phosphatases that utilize phosphoproteins as substrates have been divided, based on substrate specificity, into two major classes: the protein Ser/Thr phosphatases and the protein-tyrosine phosphatases (PTPases). Protein Ser/Thr phosphatases remove the phosphoryl group from serine/threonine residues, while PTPases dephosphorylate tyrosine residues in phosphoprotein substrates. Amino acid sequence comparisons of the catalytic domains of PTPases with the catalytic subunits of protein Ser/Thr phosphatases have shown no sequence similarity (Charbonneau et al., 1989; Cohn, 1989). This is in marked contrast to the situation with protein kinases, where tyrosine-specific and serine/threonine-specific kinases do share sequence similarity in the catalytic domains (Hanks and Hunter, 1995). Protein-tyrosine kinases and protein Ser/Thr

kinases also share a great deal of structural and mechanistic similarity (Taylor et al., 1993; Taylor et al., 1995; Johnson et al., 1996). Thus, although the two classes of protein phosphatases catalyze the same chemical reactions, the hydrolysis of phosphate monoesters, they have evolved to employ completely different strategies to accomplish the same task. While the protein Ser/Thr phosphatases are two metal ion metalloenzymes that effect catalysis by direct attack of an activated water molecule at the phosphorus atom of the substrate (Barford, 1996), the PTPases are nonmetalloenzymes that proceed through a covalent phosphocysteine intermediate during catalytic turnover (Zhang, 1997). It may be added that the structural and mechanistic features of the protein Ser/Thr phosphatases and PTPases are also distinct from those of the nonspecific acid and alkaline phosphatases and phosphatases that hydrolyze small phosphate ester molecules such as glucose 6-phosphate, fructose 1,6 bisphosphate, and inositol phosphates. The biochemical and structural properties of protein Ser/Thr phosphatases have been discussed in several recent reviews (Mumby and Walter, 1993; Shenolikar, 1994; Brautigan, 1994; and Barford, 1996).

In addition to the differences in the enzymatic mechanism, protein Ser/Thr phosphatases and PTPases also differ in their structural organization of the holoenzyme. Most protein Ser/Thr phosphatases exist as holoenzymes *in vivo* that consist of multiple subunits, while all of the PTPases are proteins of single polypeptide chain. The protein Ser/Thr phosphatase family is composed of four major types of catalytic subunits: PP1, PP2A, PP2B, and PP2C, which show broad and overlapping substrate specificity *in vitro*. It appears that the ability of protein Ser/Thr phosphatases to regulate diverse biological functions *in vivo* is due to the formation of discrete holoenzymes (Hubbard and Cohen, 1993). The combinatory association of a limited set of catalytic subunits with a large family of regulatory and targeting subunits (Campos et al., 1996) ensures the fidelity of protein Ser/Thr dephosphorylation. Interestingly, it appears that in the case of the PTPases the functional diversity within the family arises from the structural variation of regulatory and targeting sequences that are built into the same polypeptide that contains the catalytic domain.

So far, approximately 100 PTPases have been identified and the predicted total number of human PTPases may reach 500 based on genome sequencing data. Although many PTPases are proteins of greater than 400 amino acids, their catalytic domains are usually contained within a span of 250 residues. This domain is the only structural element that has amino acid sequence identity among all PTPases from bacteria to mammals (Zhang et al., 1994a). The unique feature that defines the PTPases is the active site sequence (H/V)**C**$(X)_5$**R**(S/T) in the catalytic domain called the PTPase signature motif (Zhang et al., 1994b). Traditionally, the PTPases have been categorized into receptor-like and intracellular PTPases (Figure 1). The receptor-like PTPases, exemplified by the leukocyte phosphatase, CD45 (Charbonneau et al., 1988), generally have an extracellular domain, a single transmembrane region, and one or two cytoplasmic PTPase domains. The existence of two tandem homologous PTPase domains in the receptor-like PTPases raises the interesting possibility of differential functions or regulations of the two domains. However, the significance of the repeated PTPase domain in the receptor-like PTPases is not clear. One of the important question is whether both PTPase domains in the receptor-like phosphatases are catalytically active. So far, all of the published data in the literature have indicated that the membrane proximal PTPase domain possess phosphatase activ-

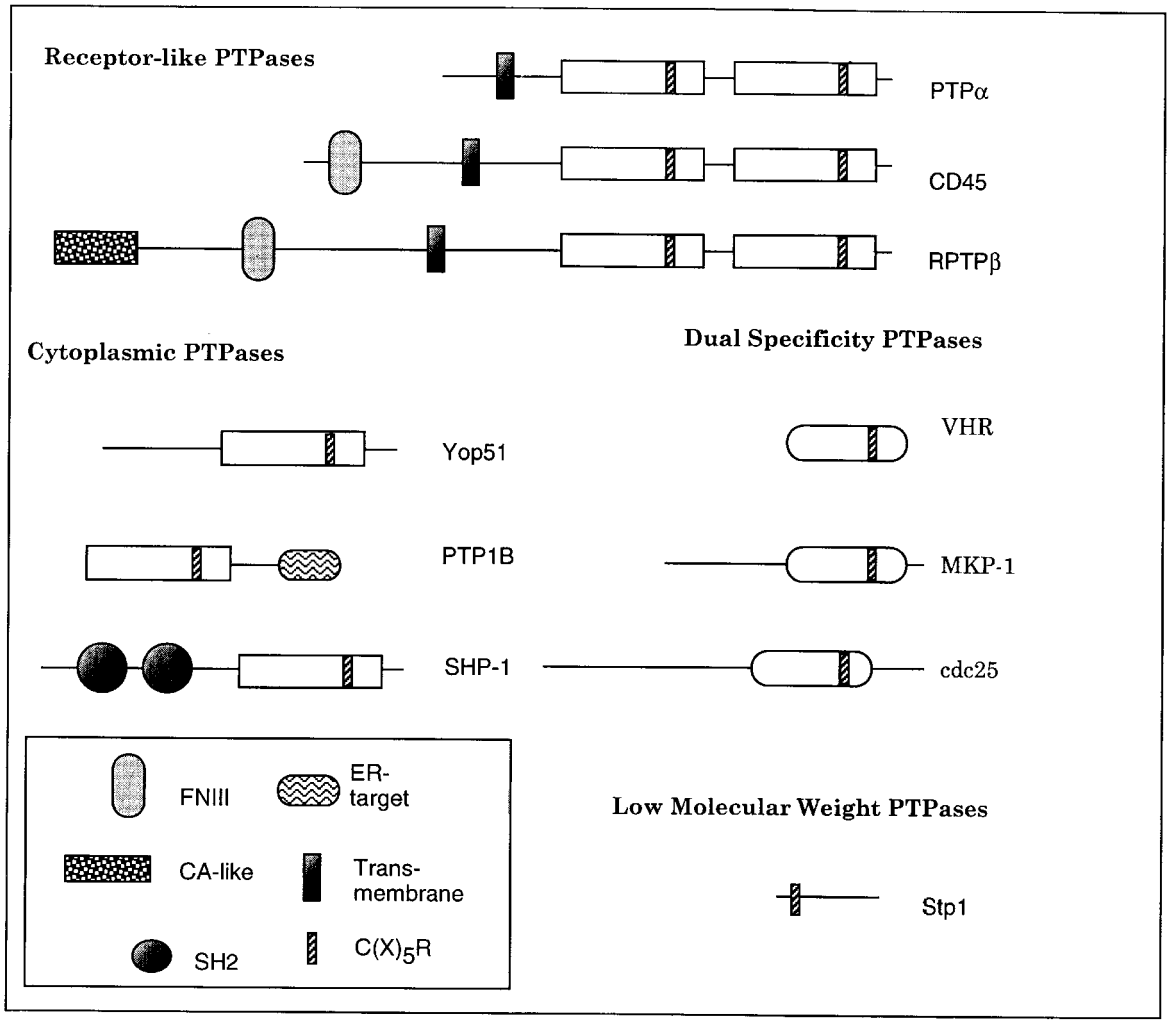

FIGURE 1. The protein-tyrosine phosphatase superfamily. FNIII, fibronectin type III, ER, endoplasmic reticulum, CA, carbonic anhydrase, SH2, src-homology 2.

ity. There is evidence that indicates that in some receptor-like PTPases the second, membrane distal PTPase domain also displays intrinsic phosphatase activity (Wang and Pallen, 1991; Wu et al., 1997; and Lim et al., 1997). However, there are also reports that suggest that the second PTPase domain is catalytically inactive and may only play, if any, a regulatory role (Streuli et al., 1989, 1990). The functional role of the second PTPase domain, that is, whether it regulates the specificity and/or activity of the first PTPase domain or exhibits independent substrate selectivity, requires further investigation. The intracellular PTPases, exemplified by PTP1B (Chernoff et al., 1990) and the *Yersinia* PTPase (Guan and Dixon, 1990), contain a single catalytic domain and various amino or carboxyl terminal extensions, including SH2 domains that may have targeting or regulatory functions. Interestingly, the PTPase signature motif can also be found in the structures of two additional groups of phosphatases that can bring about phosphotyrosine hydrolysis, namely, the VH1-like dual specificity phosphatases (Guan et al., 1991), and the low-molecular-weight (18 kDa) phos-

phatases (Zhang and Van Etten, 1990; Cirri et al., 1993) (Figure 1). The low-molecular-weight and the dual specificity phosphatases display little amino acid sequence identity with classic PTPases. The only similarities among these three groups of phosphatases are the relative placements of the essential cysteine and arginine residues in the active sites that constitute the PTPase signature motif (H/V)**C**$(X)_5$**R**(S/T). Unlike the PTPases that showed substrate specificity strictly restricted to phosphotyrosyl proteins (Sparks and Brautigan, 1985; Tonks et al., 1988b; and Guan and Dixon, 1990), the dual specificity phosphatases are unusual in that they can utilize protein substrates containing phosphotyrosine, as well as phosphoserine and phosphothreonine (Gautier et al., 1991; Guan et al., 1991; Ishibashi et al., 1992). The low-molecular-weight phosphatases display substrate specificity primarily toward phosphotyrosine, although they also show weak activities toward phosphoserine and phosphothreonine (Zhang and Van Etten, 1990; Zhang et al., 1995a).

Despite the variation in the primary structures and the differences in the active site substrate specificities, the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases utilize a common mechanism to effect catalysis (Zhang, 1997). In addition, the three-dimensional crystal structures of the catalytic domains of three tyrosine-specific phosphatases, PTP1B (Barford et al., 1994), the *Yersinia* PTPase (Stuckey et al., 1994), and PTP1α (Bilwes et al., 1996), have been determined. The three-dimensional crystal structures of a dual specificity phosphatase, VHR (Yuvaniyama et al., 1996), and the low-molecular-weight phosphatase from bovine (Su et al., 1994; Zhang, M. et al., 1994) have also been solved. Structural comparisons among these structures indicate that key structural features (e.g., the active site phosphate-binding loop formed by the (H/V)**C**$(X)_5$**R**(S/T) sequence motif) that are important for catalysis are also conserved among the three groups of phosphatases (Barford et al., 1995; Fauman and Saper, 1996). Thus, based on the similar biochemical and structural properties, the PTPase superfamily can now be grouped into three major subfamilies: the tyrosine-specific phosphatases (the classic PTPases), the dual specificity phosphatases, and the low-molecular-weight phosphatases (Figure 1). The tyrosine-specific phosphatases consist of both receptor-like and cytosolic enzymes. The dual specificity phosphatase can probably further divided into three groups based on structural comparison: (1) the VH1-like phosphatases that include VH1 (Guan et al., 1991), VHR (Ishibashi et al., 1992), and KAP/Cdi (Gyuris et al., 1993; Hannon et al., 1994), 2) the MAP kinase phosphatases (Keyse, 1995), and the cdc25 phosphatases (Sebastian et al., 1993).

The PTPase signature motif may define an even larger superfamily of phosphatases with diverse specificity. The putative mRNA capping enzyme (CEL–1) from *C. elegans* has been cloned recently and characterized (Takagi et al., 1997). The N-terminal 230 amino acids of CEL–1 is closely related to the baculovirus dual specificity phosphatase BVP (Sheng and Charbonneau, 1993) and contains the PTPase signature motif (H/V)**C**$(X)_5$**R**(S/T). Surprisingly, the recombinant amino-terminal CEL–1 display no measurable PTPase activity. As would be expected for a capping enzyme, the CEL–1 amino-terminal shows RNA triphosphatase activity, and the Cys residue in the PTPase signature motif is required for the triphosphatase activity. Interestingly, the N-terminal domains of tensin and auxilin also show amino acid sequence similarity to the PTPases, although it is not clear that they exhibit any phosphatase activity (Haynie and Ponting, 1996). Thus, it is important to characterize enzymatic activity and test for

substrate specificity of newly cloned gene products. One should not make functional assignments of newly discovered gene products based purely on sequence homology analysis.

III. BIOLOGICAL FUNCTION

A. Overview

Protein tyrosine phosphorylation, a major cellular control mechanism, is regulated by both PTKs and PTPases. The critical importance of PTKs in signaling events that control such fundamental physiological processes as cell growth and differentiation, cell cycle, and cytoskeletal reorganization have been well established. The functional role of PTPases in cellular signaling processes is just beginning to be appreciated (Sun and Tonks, 1994; Hunter, 1995; Streuli, 1996; Tonks and Neel, 1996; and Neel and Tonks, 1997). By catalyzing the removal of a phosphoryl group(s) from a tyrosine residue(s), PTPases can act both as "on" and "off" switches for signal transduction. As the natural antagonists of PTK function, PTPases can exert a negative influence on tyrosine phosphorylation-dependent signaling pathways. For example, mutations in SHP–1 leads to severe immune dysfunction, giving rise to the *moth-eaten* phenotype in mice (Shultz et al., 1993). SHP–1 is an important negative regulator of cytokine signaling; its loss resulting in sustained tyrosine phosphorylation with consequent enhanced proliferation (Klingmuller et al., 1995). The intracellular PTPase FAP–1 (Fas-associated phosphatase) binds to the carboxyl-terminal of Fas via its PDZ domains and inhibits the Fas-generated signals that lead to apoptosis (Sato et al., 1995). Overexpression of PTP1 inhibits the propagation of the IL–3 mitogenic signaling (Gelderloos and Anderson, 1996). On the other hand, PTPases can also act positively to cooperate with the PTK signaling. PTPase may assist PTK-mediated signal transduction by repriming the system for a subsequent signal or activating downstream PTKs through dephosphorylation of key tyrosine residues. Thus, the receptor PTPase CD45, through its capacity to dephosphorylate and activate the *src* family of PTKs, is essential for initiating downstream signaling processes in response to stimulation of T and B cell receptors (Pingel and Thomas, 1989). SHP–2 and its *Drosophila* homolog *corkscrew* are positive mediators of growth factor signaling (Perkins et al., 1992; Noguchi et al., 1994). The cell cycle regulator cdc25 dephosphorylates Thr14 and Tyr15 of cdc2, thereby activating the cdc2/cyclin B complex, which, in turn, promotes mitosis (Gould and Nurse, 1989; Millar and Russell, 1992).

Since the discovery of the *vaccinia* VH1 phosphatase, a number of additional dual-specificity phosphatases have been identified. The mammalian dual specificity protein phosphatases have surfaced recently as key regulators of mitogenic signaling pathways as well as the cell cycle itself (Sun et al., 1993; Aroca et al., 1995; and Keyse, 1995). They include the cell cycle regulator cdc25 (Gautier et al., 1991), KAP (Hannon et al., 1994) or Cdi (Gyuris et al., 1993), and a number of mitogen-activated kinases (MAP kinases) phosphatases (Sun et al., 1993; Keyse, 1995; Chu et al., 1996). While cdc25 activates the cyclin-dependent kinases (Gautier et al., 1991), KAP/Cdi inactivates them (Poon and Hunter, 1995). A number of mammalian dual specificity protein phosphatases have been shown to be capable of dephosphorylating and inactivating mitogen-activated kinases (MAP kinases) (Sun et al., 1993; Keyse, 1995; Chu et al., 1996). The low-molecular-weight PTPases, whose biological function is unknown, were previ-

ously found only to exist in mammalian species (Ramponi, 1994). Genetic studies have demonstrated that the fission yeast (*Schizosaccharomyces pombe*) homologue of the mammalian low-molecular-weight phosphatase, Stp1, is a multicopy repressor of cdc25, indicating a role in cell cycle regulation (Mondesert et al., 1994). However, neither disruption of the *Saccharomyces cerevisiae* homologue (LTP1) gene nor an overexpression of its product in the yeast cause any apparent phenotypic changes (Ostanin et al., 1995), suggesting functional redundancy *in vivo*.

B. PTPases and Cancers

The level of tyrosine phosphorylation, and thus the strength and duration of the signal transmitted, are balanced by the opposing action of PTKs and PTPases. This balance is perturbed in transformed cells and metabolic disorders. Because deregulated PTKs, such as *src*, *lck*, and *neu*, can function as dominant oncogenes (Cantley et al., 1991), it has been expected that, at least some PTPases, function as products of tumor suppressor genes. This expectation has certainly been met by two reports that describe the cloning and characterization of the first tumor suppressor gene encoding a phosphatase in the PTPase superfamily. This gene, called *PTEN* (*Phosphatase and Tensin homologue deleted on Chromosome 10*, Li et al., 1997) or *MMAC1* (*Mutated in Multiple Advanced Cancers 1*, Steck et al., 1997), resides on chromosome 10q23 and is mutated in several major groups of sporadic cancers, including brain, breast, and prostate cancers. Germline mutations of the *PTEN/MMAC1* gene have also been linked to an inherited autosomal-dominant cancer syndrome, Cowden's syndrome, that is associated with benign lesions of the skin, breast, and thyroid (Liaw et al., 1997). The gene for the receptor-like PTPγ has also been proposed to be a tumor suppressor gene, which is located on chromosome 3p21, a segment frequently altered in renal and lung carcinomas (LaForgia et al., 1991; Wary et al., 1993). In addition, expression of PTP1B has been shown to block transformation mediated by *neu* (Brown-Shimer et al., 1992) and partially revert transformation by *src* (Woodford-Thomas et al., 1992).

Interestingly, there is mounting evidence that some PTPases can potentiate, rather than antagonize, the action of PTKs. This behavior enhances mitogenic signaling and can lead to cellular transformation. For example, cdc25A, a member of the cdc25 family, functions at the start of the cell cycle and regulates the G1/S-phase transition (Hoffmann et al., 1994). Cdc25A cooperates with Ha-ras in the oncogenic transformation of primary rodent fibroblasts and is overexpressed in primary breast tumors, suggesting that they are human oncogenes (Galaktionov et al., 1995, 1996). Evidence suggest that cdc25A is a target of the protooncogene c-*myc* (Galaktionov et al., 1996), which belongs to a family of related genes implicated in the control of normal cell proliferation and the induction of neoplasia (Bishop, 1983). In addition, cdc25A is also a target of the adenovirus E1A oncoprotein (Spitkovsky et al., 1996). E1A interferes with cellular growth control leading to a deregulation of the cell cycle control machinery (Hunter and Pines, 1994). Cdc25A phosphatase activity as well as cdc25A gene expression are strongly increased after expression of E1A in quiescent human fibroblasts. Moreover, microinjection of specific antibodies against cdc25A blocks virus-induced S-phase entry, indicating that cdc25A function is required for adenovirus-mediated cell proliferation (Spitkovsky et al., 1996). Strikingly, ectopic expression of PTPα produces a trans-

formed phenotype in rat embryonic fibroblasts, and the PTPα overexpressing cells are tumorigenic *in vivo*, forming tumors in nude mice 5 to 6 d after injection (Zheng et al., 1992). Evidence suggests that PTPα is involved in the pathway that leads to the activation of c-*src*, thereby potentiating mitogenic signals (Zheng et al., 1992; den Hertog et al., 1993). In addition, increased mRNA expression of PTPα has been detected in late-stage colon carcinomas (Tabiti et al., 1995). Its oncogenic properties suggest that PTPα overexpression could contribute to the tumorigenic process in colon cancer. The rat nuclear PTPase, PRL–1, is an immediate-early gene in liver regeneration and is positively associated with growth, including fetal and neoplastic hepatic growth and anchorage-independent growth after overexpression in fibroblasts (Diamond et al., 1994). Indeed, overexpression of the human homologs of rat PRL–1 (PTPCAAX1 and PTPCAAX2) in epithelial cells caused a transformed phenotype in culture and tumor growth in nude mice (Cates et al., 1996). Interestingly, the plant oncogene *rolB*, from *Agrobacterium rhizogenes*, has been shown recently to encode a protein that displays PTPase activity (Filippini et al., 1996).

C. PTPases and Diabetes

Tyrosine phosphorylation is a key reaction in the initiation and propagation of insulin action (White and Kahn, 1994; Myers and White, 1996). Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with intrinsic PTK activity. Insulin receptor is activated after insulin binding and undergoes autophosphorylation, leading to the subsequent tyrosine phosphorylation of intracellular proteins. Predominate among these phosphorylated proteins in most cell types is the protein known as insulin receptor substrate 1. The phosphorylation on tyrosine residues in insulin receptor and insulin receptor substrate 1 generate docking sites for other enzymes and effector molecules containing SH2 domains or phosphotyrosine-binding domains to propagate the insulin signal. The termination of insulin action, after insulin withdrawal, consequently requires the dephosphorylation of both insulin receptor and insulin receptor substrate 1. Several PTPases, including PTP1B (Ahmad et al., 1995; Kenner et al., 1996; Kole et al., 1996), LAR (Kulas et al., 1995; Zhang, W.-R. et al., 1996), and PTPα (Moller et al., 1995; Lammers et al., 1997), have been implicated as negative regulators of the insulin receptor signaling. Overexpression of these PTPases in cell cultures decreases the insulin-stimulated receptor and/or insulin receptor substrate 1 phosphorylation, while reduction of the level of the active PTPases by antisense RNA, neutralizing antibodies, or inhibitors augments the insulin initiated signaling pathways. This is consistent with the well-known insulin-mimetic effect of vanadate (Heyliger et al., 1985; Goldfine et al., 1995), which is a general inhibitor of PTPases (Swarup et al., 1982; Huyer et al., 1997). It is observed that the $G_{i\alpha 2}$ deficiency animal model exhibits insulin resistance characteristic of non-insulin-dependent diabetes mellitus (NIDDM). The physiological defect in NIDDM is insulin resistance, which is the inability of insulin to stimulate glucose transport from blood to muscle. Interestingly, $G_{i\alpha 2}$ deficiency increases PTPases activity and attenuates insulin-stimulated tyrosine phosphorylation of insulin-receptor substrate 1 *in vivo*, implicating PTPase as a critical link between $G_{i\alpha 2}$ deficiency and insulin resistance (Maxham et al., 1996). In addition, insulin resistance in the ob/ob mouse model is associated with a reduction in insulin-induced protein-tyrosine phosphorylation, possibly due to increased PTPase

activity (Sredy et al., 1995). Thus, the development of specific inhibitors of PTPases acting on the insulin receptor or its endogenous substrates could enhance insulin action in human disease states with insulin resistance such as NIDDM.

D. PTPases and Infectious Diseases

PTPases may represent novel targets for antibiotics development. A number of bacterial and viral pathogens have evolved sophisticated strategies to subvert host-cell signal-transduction pathways for their own benefit. For instance, the pathogenic bacteria *Yersinia* encodes a PTPase essential for its virulence. The genus *Yersinia* comprises three species of bacteria that are causative agents in human diseases ranging from gastrointestinal syndromes to the Bubonic Plague (Butler, 1985). *Yersinia pestis* is the pathogen responsible for the Bubonic Plague, also known as the Black Death, because it reduced the population of Europe by some 25 million in the 15th century (Butler, 1985). Credible estimates of the number of people killed by this bacterium during the course of human history approach 200 million (Brubaker, 1991). Although plague has long been considered a once-vanquished disease, the recent outbreak of the pneumonic plague caused by *Yersinia pestis* in Surat, India (Jayaraman, 1994), proves that its biological potential can be expressed under appropriate environmental conditions. In addition, *Yersinia enterocolitica* infection still poses a serious problem in public health (Cover and Aber, 1989). The *Yersinia* PTPase, identified in the genus *Yersinia* (Guan and Dixon, 1990), is obligatory for pathogenesis (Bolin and Wolf-Watz, 1988; Michielis and Cornelis, 1988). Furthermore, it has been demonstrated that the phosphatase activity of the *Yersinia* PTPase is essential for virulence (Bliska et al., 1991). Because bacteria are generally believed to contain no tyrosine phosphorylated proteins (Foster et al., 1989), the targets for the *Yersinia* PTPase are likely host proteins. Indeed, infection of a macrophage cell line resulted in specific dephosphorylation of only two cellular protein substrates (Bliska et al., 1992). One of the substrates of the *Yersinia* PTPase in human epithelial cell was identified as $p130^{cas}$ (Black and Bliska, 1997). $p130^{cas}$ is a novel docking protein localized to focal adhesions and is phosphorylated on numerous Tyr residues in response to integrin engagement (Sakai et al., 1994; Harte et al., 1996). This may explain the ability of the *Yersinia* PTPase to promote cell detachment from the extracellular matrix (Bliska et al., 1993). Interestingly, the bacterial pathogen *Salmonella typhimurium,* which causes Typhoid fever and a variety of food poisonings, secretes a PTPases required for its full display of virulence (Kaniga et al., 1996). The *vaccinia* virus encodes a dual specificity phosphatase, VH1, that is essential for viral transcription and infectivity (Liu et al., 1995). In addition, the Myxoma virus and Shope Fibroma virus also encode dual specificity phosphatases that are essential factors for virus viability (Mossman et al., 1995). Finally, PTPase activity has also been detected in parasites such as *Leishmania donovani* (Cool and Blum, 1993), *Trypanosoma brucei*, and *Trypanosoma cruzi* (Bakalara et al., 1995a,b). These results underscore the importance of PTPase regulation in normal cellular function.

IV. STRUCTURE

A. Overview

The tyrosine-specific phosphatases (the classic PTPases), the VH1-like dual specificity phosphatases and the low-molecular-weight phosphatases share little amino acid

sequence identity. The amino acid sequence alignment for the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases are shown in Figure 2. As can be seen, the only similarities among these three groups of phosphatases are the relative placements of the essential cysteine and arginine residues in the active sites that constitute the PTPase signature motif (H/V)**C**$(X)_5$**R**(S/T) (Figures 2 and 3).

The three-dimensional structures of the PTPase catalytic domains of PTP1B (Barford et al., 1994; Jia et al., 1995) and the *Yersinia* PTPase (Stuckey et al., 1994; Schubert et al., 1995; Fauman et al., 1996) have been reported. The X-ray structure of the membrane proximal PTPase domain (D1) in PTPα has also been determined (Bilwes et al., 1996). Although the amino acid sequence of the *Yersinia* PTPase is only ~20% identical to the mammalian PTPases, it is clear that the three structures share a common secondary structural scaffold, with close similarity in tertiary structure. The PTPases are α + β proteins with tertiary folds composed of a highly twisted mixed β-sheet flanked by α-helices on both sides (Figure 4). The three-dimensional structure of the dual specificity phosphatase VHR reveals a general fold that occurs in the *Yersinia* PTPase, PTP1B, and PTPα structures, and many of the secondary structural elements of PTPases are also present in VHR (Figure 4). Although VHR is smaller than PTPases, the VHR structure retains the same starting and ending secondary structure elements as the PTPase catalytic domains, suggesting that the VHR structure may define a minimal core structure for both dual specificity phosphatases and the PTPases (Yuvaniyama et al., 1996). In addition to the general lack of sequence identity between the low-molecular-weight phosphatases and the PTPases, the low-molecular-weight enzymes are also relatively smaller in size and contain PTPase signature motif close to the NH_2-terminus of the protein, whereas the signature motif occurs closer to the COOH-terminus of the PTPases and the dual specificity phosphatases. The bovine low-molecular-weight phosphatase has distinct topologies compared with those of PTP1B, the *Yersinia* PTPase, PTPα, and VHR (Su et al., 1994; Zhang, M. et al., 1994; Logan et al., 1994; Zhou et al., 1994). However, it is interesting that residues of the PTPase signature motif of the PTPases, the dual specificity phosphatases and the low-molecular-weight phosphatases form a similar loop structure that is defined here as the PTP loop that binds the phosphoryl moiety of the substrate. The PTP loop is located between the β-turn at the COOH-terminus of a β strand and the first turn of an α helix. Furthermore, structural comparison between the bovine low-molecular-weight phosphatase and PTPases and VHR reveals that the structures of these phosphatases display a similar arrangement of secondary structure elements, albeit with somewhat different connectivity (Barford et al., 1995; Fauman and Saper, 1996). Thus, these different phosphatase structures are striking examples of convergent evolution achieving highly similar active site clefts, and the similarities in the conserved active site motifs may suggest a common mechanism to bring about phosphate monoester hydrolysis in these otherwise very different molecules. The detailed structural properties of these phosphatases have been reviewed (Barford et al., 1995; Zhang, M. et al., 1995; Fauman and Saper, 1996; Zhang, 1997). Only the salient features that are pertinent to PTPase catalysis are discussed below.

B. The PTP-loop

The phosphatase active site is located within a crevice (~9 Å deep for the tyrosine-specific phosphatases and ~6 Å deep for VHR) on the molecular surface. The central

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 Yop51  APATNDPRYL QACGGEKLNR FRDIQCCRQT AV........ ..RAD.LNAN YIQVGNTRT.
hTcell  ESHDYPHRVA KFPENRNRNR YRDVSPYDHS RVKL.....Q NAENDYINAS LV.....DIE
 rPTP1  EASDFPCRIA KLPKNKNRNR YRDVSPFDHS RIKL.....H QEDNDYINAS LI.....KME
hPTP1C  VKNLHQRLEG QRPENKGKNR YKNILPFDHS RVILQGRDSN IPGSDYINAN YIKNQLLGPD
  rLAR  ...QFTWENS NSEVNKPKNR YANVIAYDHS RVLL.TSIDG VPGSDYINAN YI.....DGY
 Mega1  .KPGMTMSCA KLPQNISKNR YRDISPYDAT RVIL...... KGNEDYINAN YI.NMEIPSS
   PEP  ADKIYPTTVA QRPKNIKKNR YKDILPYDHS LVEL.SLLTS DEDSSYINAS FI.....KGV

 Yop51  ......IACQ YPLQSQLESH FRMLAENRTP VLAVLASSSE IANQRFGMPD YFRQSGTYGS
hTcell  EAQRSYILTQ GPLPNTCCHF WLMVWQQKTK AVVMLNRIVE KESVKCAQ.. YWP.TD.DQE
 rPTP1  EAQRSYILTQ GPLPNTCGHF WEMVWEQKSR GVVMLNRIME KGSLKCAQ.. YWPQKE.EKE
hPTP1C  ENAKTYIASQ GCLEATVNDF WQMAWQENSR VIVMTTREVE KGRNKCVP.. YWPEVG.MQR
  rLAR  RKQNAYIATQ GPLPETMGDF WRMVWEQRTA TVVMMTRLEE KSRVKCDQ.. YWPARG.TE.
 Mega1  SIINQYIACQ GPLPHTCTDF WQMTWEQGSS MVVMLTTQVE RGRVKCHQ.. YWPEPT.GSS
   PEP  YGPKAYIATQ GPLSTTLLDF WRMLWEYRIL VIVMACMEFE MGKKKCER.. YWAEPG.ETQ
                                                              *
 Yop51  ITVESKMTQQ VGLGDG.... .IMADMYTLT IREAGQKTIS VPVVHVGNWP DQTAVSSEV.
hTcell  MLFKETGFSV KLLSEDVKSY YTVHLLQLEN INS.GE.TRT ISHFHYTTWP D.FGVPE..S
 rPTP1  MVFDDTNLKL TLISEDVKSY YTVRQLELEN LAT.QE.ARE ILHFHYTTWP D.FGVPE..P
hPTP1C  AY...GPYSV TNCGEHDTTE YKLRTLQVSP LDN.GDLIRE IWHYQYLSWP D.HGVPS..E
  rLAR  TY...GLIQV TLVDTVELAT YTMRTFALHK SGS.SE.KRE LRQFQFMAWP D.HGVPE..Y
 Mega1  SY...GCYQV TCHSEEGNTA YIFRKMTLFN QEK.NE.SRP LTQIQYIAWP D.HGVPD..D
   PEP  LQF..GPFSI SCEAEKKKSD YKIRTLKAKF N...NE.TRI IYQFHYKNWP D.HDVPS..S
                                                 *      **
 Yop51  TKALASLVDQ TAETKRNMYE SKGSSAVADD SKLRP.VIHC RAGVGRTAQL IGAMCM....
hTcell  PASFLNFLFK VRESGSLNPD HG........ ....PAVIHC SAGIGRSGTF SLVDTCLVLM
 rPTP1  ASFLNFLSFK VRESGSLSPE HG........ ....PIVVHC SAGIGRSGTF CLADTCLLLM
hPTP1C  PGGVLSFLDQ INQRQESLPH AG........ ....PIIVHC SAGIGRTGTI IVIDMLMENI
  rLAR  PTPILAFLRR VKACNP..LD AG........ ....PMVVHC SAGVGRTGCF IVIDAMLERM
 Mega1  SSDFLDFVCH VRNKRAG..K EE........ ....PVVVHC SAGIGRTGVL ITMETAMCLI
   PEP  IDPILQLIWD MRCYQE..DD CV........ ....PICIHC SAGCGRTGVI CAVDYTWMLL

 Yop51  ..NDSRNSQL SVEDMVSQMR VQRNGIMVQK DEQLDVLIKL AEGQGRPLLN S....
hTcell  EKGDD...IN .IKQVLLNMR KYRMG.LIQT PDQLRFSYMA IIEGAKCIKG DSSIQ
 rPTP1  DKRKDPSSVD .IKKVLLEMR RFRMG.LIQT ADQLRFSYLA VIEGAKFIMG DSSVQ
hPTP1C  STKGLDCDID .IQKTIQMVR AQRSG.MVQT EAQYKFIYVA IAQFIETTKK KLEVL
  rLAR  KH...EKTVD .IYGHVTCMR SQRNY.MVQT EDQYVFIHEA L..LEAAMCG HTEVL
 Mega1  ECNQPVYPLD .I...VRTMR DQRAM.MIQT PSQYRFVCEA ILKVYEEGFV KPLTT
   PEP  KDGIIPKNFS .VFNLIQEMR TQRPS.LVQT QEQYELVYSA VLELFKRHMD VISDN
```

A

FIGURE 2. Amino acid sequence alignments of (A) PTPases, (B) dual specificity phosphatases, and (C) low-molecular-weight phosphatases. The catalytic essential Cys, Arg, and Ser/Thr in the PTPase signature motif, and the general acid/base Asp residue are highlighted by an asterisk (*).

feature of these structures is a strand-loop-helix element where the loop (PTP loop) contains the PTPase signature motif ((H/V)**C**(X)$_5$**R**(S/T)). Oxyanions such as phosphate, tungstate, or sulfate bind within this PTP loop (Figure 5). The active site pocket is formed by the PTP loop at its base and surrounded by loops that provide an essential Asp general acid and residues that interact with the pTyr or pSer/pThr in peptide substrates (Figure 5). The crystal structures of the phosphatases complexed with oxyanions provide information about the interactions between the enzyme and the

```
KAP  MKPPSSIQTS EFDSSDEEPI EDEQTPIHIS WLSLSRVNCS QFLGLCALPG CKFKDVRRNV
VH1  .......... .......... .MDKKSLYKY LLLRSTGDMH KAKSPTIMTR VTNNVYLGNY
VHR  .......... .....MSGSF ELSVQDLNDL L...SDGSGC YSLPSQPCNE VTPRIYVGNA

                                                              *
KAP  QKDTEELKSC GIQDIFVFCT RGELSKYRVP NLLDLYQQCG ITHHHPIADD GGTPDIASCC
VH1  KNAMDAPSSE .VKFKYVLNL TMDKYTLP.. .......NSN INIIHIPLVD DTTTDISKYF
VHR  SVAQDIPKLQ KLGITHVLNA AEGRSFMHVN TNANFYKDSG ITYLGIKAND TQEFNLSAYF

                              *     **
KAP  EIMEELTTCL KNYRK..TLI HCYGGLGRSC LVAACLLLYL SDTISPEQAI DSLRDLRGSG
VH1  DDVTAFLSKC .DQRNEPVLV HCAAGVNRSG AMILAYLM.S KNKESSPMLY FLYVYHSMRD
VHR  ERAADFIDQA LAQKNGRVLV HCREGYSRSP TLVIAYLM.M RQKMDVKSAL SIVRQNREIG


KAP  AIQTIKQYNY LHEFRDKLAA HLSSRDSQSR SVSR
VH1  LRGAFVENPS F..KRQIIEK YVIDKN
VHR  PNDGFLAQLC Q..LNDRLAK EGKLKP
```

FIGURE 2B

```
                                  *     * *
human(s)              AEQATKSV LFVCLGNICR SPIAEAVFRK LVTDQNISEN W.VIDSGAVS
human(f)              AEQATKSV LFVCLGNICR SPIAEAVFRK LVTDQNISEN W.RVDSAATS
bovine                AEQVTKSV LFVCLGNICR SPIAEAVFRK LVTDQNISDN W.VIDSGAVS
S. pombe               MTKNIQV LFVCLGNICR SPMAEAVFRN EVEKAGLEAR FDTIDSCGTG
S. cerevisiae         IEKPKISV AFICLGNFCR SPMAEAIFKH EVEKANLENR FNKIDSFGTS
S. coelicolor         AEQMTYRV CFVCTGNICR SPMAEAVFRA RVEDAGL.GH LVEADSAGTG


human(s)           DWNVGRSPDP RAVSCLRNHG IHTAHKARQI TKEDFATFDY ILCMDESNLR
human(f)           GYEIGNPPDY RGQSCMKRHG IPMSHVARQI TKEDFATFDY ILCMDESNLR
bovine             DWNVGRSPDP RAVSCLRNHG INTAHKARQV TKEDFVTFDY ILCMDESNLR
s. pombe           AWHVGNRPDP RTLEVLKKNG IHTKHLARKL STSDFKNFDY IFAMDSSNLR
S. cerevisiae      NYHVGESPDH RTVSICKQHG VKINHKGKQI KTKHFDEYDY IIGMDESNIN
S. coelicolor      GWHEGEGADP RTEAVLADHG YGLDHAARQF QQSWFSRLDL VVALDAGHLR

                                                          *
human(s)           DLNRKSNQVK TCKAKIELLG SY...DPQKQ LIIEDPYYGN DSDFETVYQQ
human(f)           DLNRKSNQVK TCKAKIELLG SY...DPQKQ LIIEDPYYGN DSDFETVYQQ
bovine             DLNRKSNQVK NCRAKIELLG SY...DPQKQ LIIEDPYYGN DADFETVYQQ
s. pombe           NINRVKPQ.. GSRAKVMLFG EYA..SPGVS KIVDDPYYGG SDGFGDCYIQ
S. cerevisiae      NL.KK.IQPE GSKAKVCLFG DWNTNDGTVQ TIIEDPWYGD IQDFEYNFKQ
S. coelicolor      ALRRLAPTER DA.AKVRLLR SYDPAVAGGD LDVPDPYYGG RDGFEECLEM


human(s)           CVRCCRAFLE KAH
human(f)           CVRCCRAFLE KAH
bovine             CVRCCRAFLE KVR
s. pombe           LVDFSQNFLK SIA
S. cerevisiae      ITYFSKQFLK KEL
S. coelicolor      VEAASTGLLA AVREQVEGRAA
```

FIGURE 2C

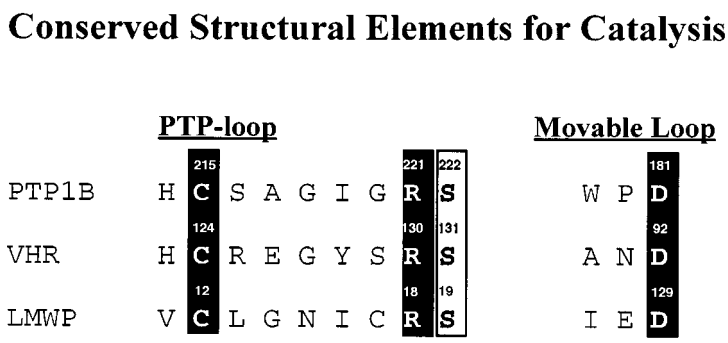


FIGURE 3. Conserved structural elements for catalysis by the PTPases, the dual specificity phosphatases and the low-molecular-weight phosphatases.

phosphoryl moiety of the substrate. The oxygen atoms of the oxyanion make two hydrogen bonds with the guanidinium group of the invariant Arg residue in the PTP-loop. Kinetic studies have demonstrated that the invariant Arg residue in the signature motif plays an important role in substrate binding and transition state stabilization (Zhang et al., 1994b). The oxygen atoms of the oxyanion are also hydrogen bonded to the NH amides of the peptide backbone making up the PTP-loop. Thus, it is likely that the side chain of the Arg residue acts together with the amides of the PTP loop to ligand the phosphoryl group in a substrate.

The invariant Cys residue in the PTP loop has been shown to be essential for phosphatase activity and formation of a covalent cysteinyl phosphoenzyme intermediate (Zhang, 1990; Guan and Dixon, 1991; Wo et al., 1992; Cho et al., 1992; and Zhou et al., 1994). The PTPase-tungstate complexes reveal that the Sγ atom of Cys403 in the *Yersinia* PTPase is poised 3.6 Å from the W atom, while the Sγ atom of Cys215 in PTP1B is poised 3.1 Å from the W atom. In the catalytic inactive mutant PTP1B/C215S-substrate complexes, the Oγ of Ser215 is situated within the center of the PTP loop, 3.2 Å from the phosphorous atom of pTyr such that the Oγ of Ser215, the phosphorous of pTyr, and the phenolic oxygen in tyrosine are approximately co-linear (Jia et al., 1995). This is consistent with the side chain of the Cys residue acting as a nucleophile in the catalytic mechanism, as discussed below. The apparent thiol pK_a of the active site Cys residue was found to be 4.7 in the *Yersinia* PTPase (Zhang and Dixon, 1993), 5.4 in PTP1 (Lohse et al., 1997), and 5.6 in VHR (Denu et al., 1995), indicating that the Cys exists as a thiolate anion at physiological pH. Unlike the cysteine proteinases, which stabilize an active site thiolate anion via an ion-pair with a protonated histidine, the PTPases likely stabilize the thiolate by a number of structural elements in the active site. The Cys Sγ is at the center of the PTP loop and within 3 to 4 Å from every amide nitrogen of the PTP loop. The orientation of these microdipoles is essential for phosphate binding and thiolate stabilization. The positioning of the macrodipole generated by the major helix (α5 in *Yersinia* PTPase and α4 in PTP1B) is expected contribute to the stability of the anion. Finally, the guanidinium group in the Arg residue and the hydroxyl group in the Ser/Thr residue of the PTP loop may also help to lower the thiol pK_a (Barford et al., 1994; Zhang et al., 1995b).

C. The Movable Loop

Biochemical studies have demonstrated the involvement of an essential Asp residue

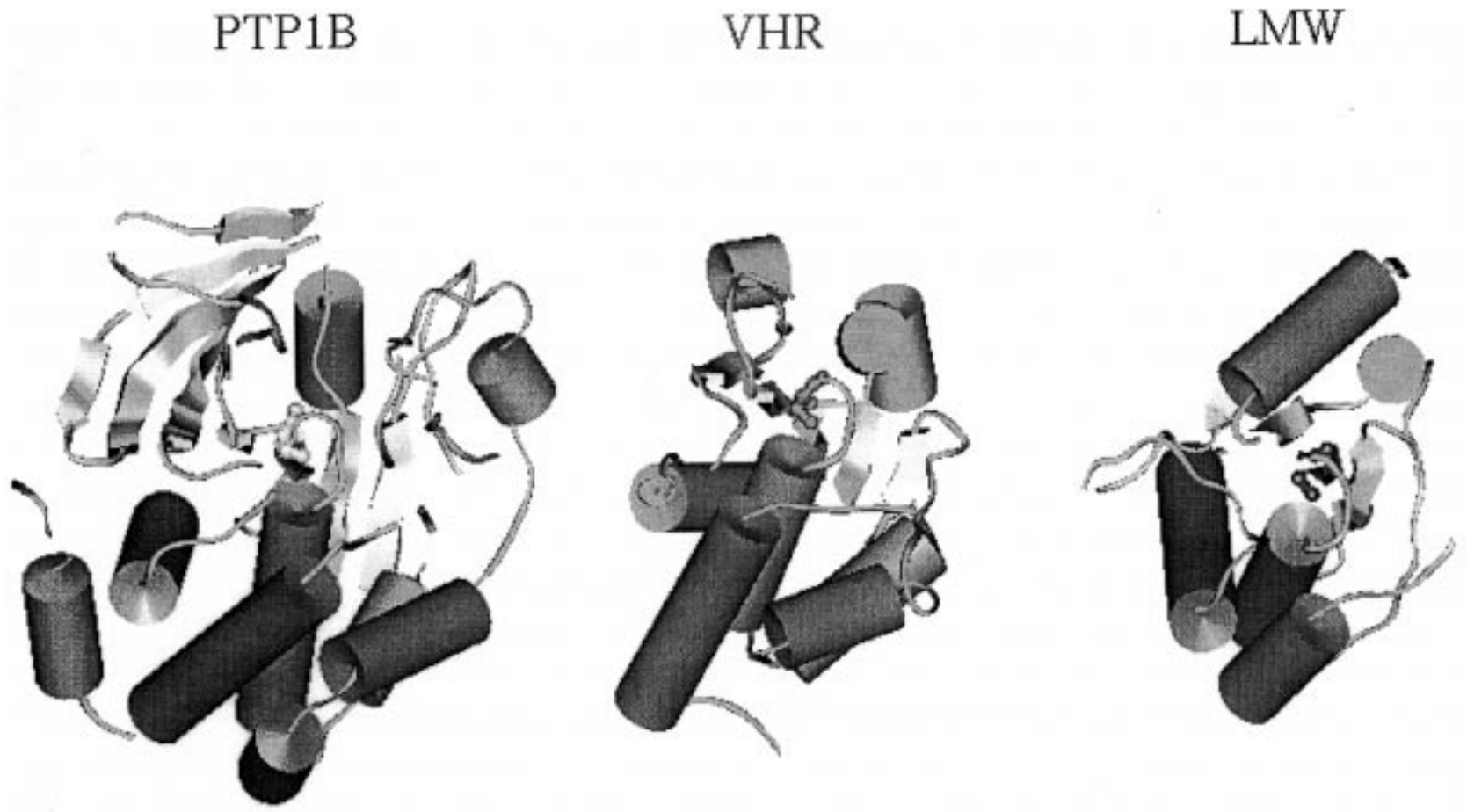


FIGURE 4. Overall, three-dimensional folds of PTP1B (Barford et al., 1994), VHR (Yuvaniyama et al., 1996), and the bovine low-molecular-weight phosphatase (Su et al., 1994). The active site Cys residue is highlighted for orientational purposes.

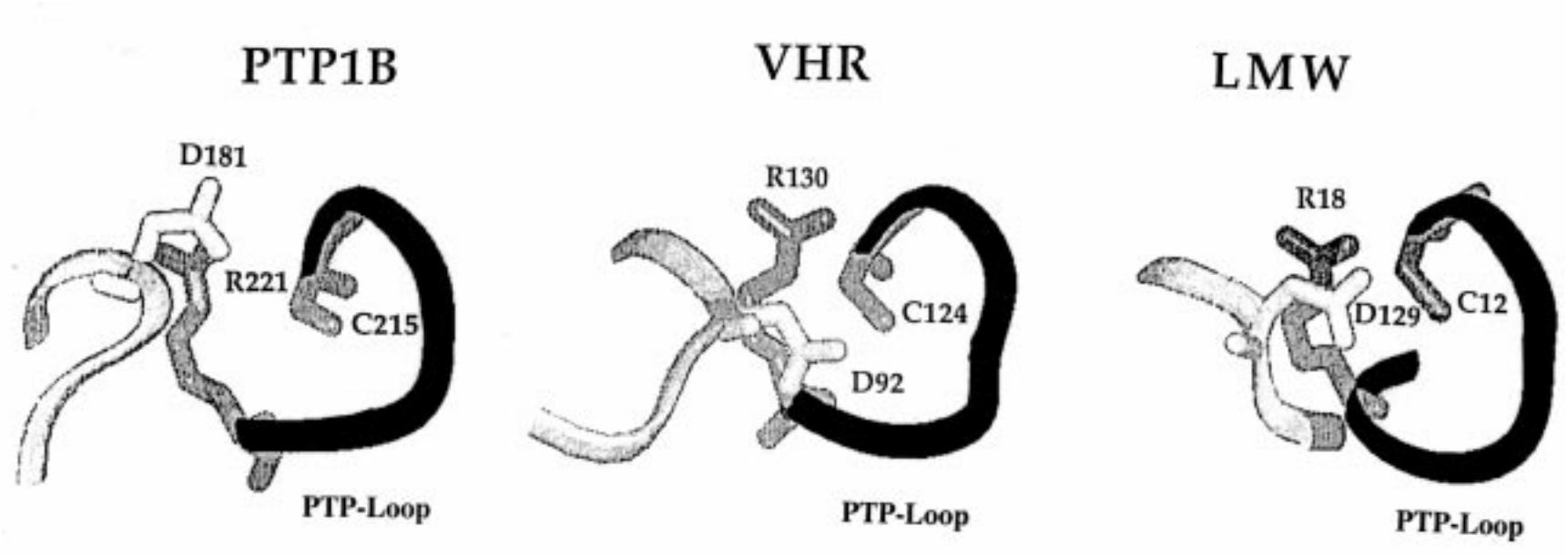


FIGURE 5. Active site conformation highlighting the PTP loop and the movable loop in PTP1B, VHR, and the bovine low-molecular-weight phosphatase. The black ribbon indicates the PTP loop with the cysteine nucleophile positioned for attack. The invariant nucleophilic cysteine, the general acid aspartic residue, and the binding site arginine are labeled. The PTP1B structure was solved with peptide substrate (Jia et al., 1995). The VHR and the low-molecular-weight phosphatase structures were solved with bound sulfate or phosphate (Su et al., 1994; Yuvaniyama et al., 1996).

Yersinia PTPase	H	V	G	N	**W**	P	**D**	Q	T	A	V	P
Salmonella ptpase	H	V	K	N	**W**	P	**D**	H	Q	P	L	P
Yeast PTP1	Y	F	D	L	**W**	K	**D**	M		N	K	P
Yeast PTP2	Q	Y	K	N	**W**	L	**D**	S	C	G	V	D
Human PTP1B	H	Y	T	T	**W**	P	**D**	F		G	V	P
Human PTP-PEST	H	Y	V	N	**W**	P	**D**	H		D	V	P
Human SHP-1	Q	Y	L	S	**W**	P	**D**	H		G	V	P
Human PTPmega1	Q	Y	I	A	**W**	P	**D**	H		G	V	P
Human CD45 (D1)	Q	F	T	S	**W**	P	**D**	H		G	V	P
Human LAR (D1)	Q	F	M	A	**W**	P	**D**	H		G	V	P

FIGURE 6. Amino acid sequence comparison of the WpD loop (residues 350–361 in the *Yersinia* PTPase) in a number of PTPases.

as the general acid in catalysis (for detail see the section on General Acid/Base Catalysis). The crystal structures of the phosphatase-oxyanion complexes reveal that the catalytic Asp residue, located on a loop adjacent to the PTP loop but opposite the nucleophilic cysteine, is pointed toward the bound oxyanion (Figure 5, Stuckey et al., 1994; Su et al., 1994; Zhang, M. et al., 1994; and Yuvaniyama et al., 1996). The carboxylate is 2.7 to 3.8 Å away from the oxyanion oxygen, depending on the pH of crystallization, which is structurally homologous to the scissile oxygen of a phosphotyrosine substrate. Indeed, in the structure of PTP1B/C215S-substrate complex, Asp181 forms a network of hydrogen bonds to the phenolic oxygen of phosphotyrosine and a buried water molecule (Jia et al., 1995). Thus, the Asp residue is ideally positioned to donate a proton to the tyrosine leaving group during the first hydrolysis step.

Structure superpositions of the ligand-free and ligand-bond PTPases indicate that the only prominent conformational differences reside in the surface loop that harbors the general acid Asp residue. For PTPases, the sequence of this loop, termed WpD loop, is quite divergent except for the WpD sequence that includes the catalytic aspartic acid and a tryptophan near the hinge position of the loop (Figure 6). It was observed that in the unliganded *Yersinia* PTPase structure, Asp356 is greater than 10 Å from the phosphate binding site. In the oxyanion-complexed structures, the surface loop (residues 351 to 360) that contains the Asp356 residue has moved like a "flap" to cover the active site (Stuckey et al., 1994; Schubert et al., 1995). The C_α of Asp356 itself moves 6 Å toward the active site after tungstate binding, positioning its carboxylate close to the oxyanion oxygen that is structurally homologous to the scissile oxygen of a phosphotyrosine substrate (Figure 7). Similarly, binding of either phosphotyrosine or phosphotyrosine-containing peptide to the Cys215 to Ser mutant mammalian PTPase, PTP1B, induces a conformational change of an equivalent surface loop that brings the corresponding Asp181 into the catalytic site and forms a network of hydrogen bonds to the phenolic oxygen of phosphotyrosine and a buried water molecule (Jia et al., 1995). Thus, substrate binding causes a conformational change of the WpD loop that brings

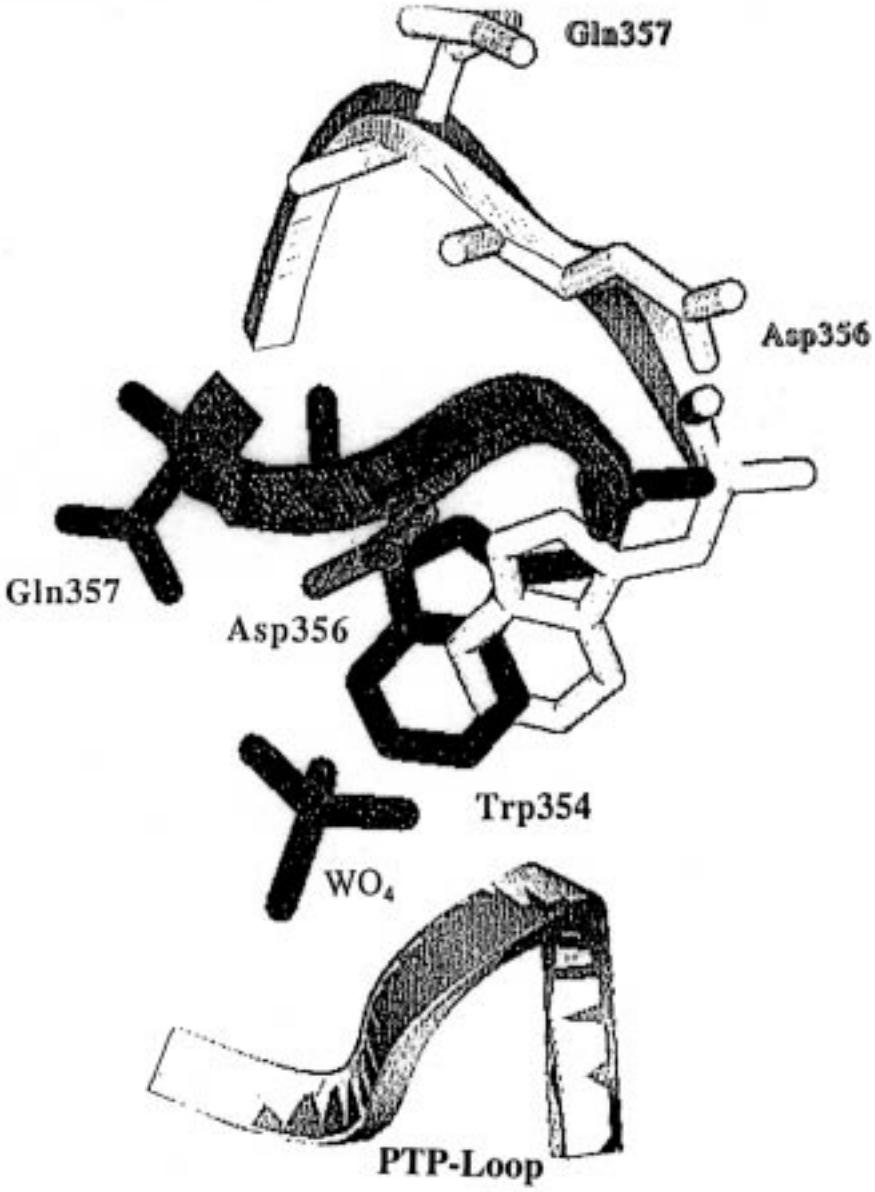


FIGURE 7. Conformational change in the WpD loop. In the *Yersinia* PTPase, conformational change in the WpD loop moves Asp356 into the active site and in hydrogen bonding distance from the tungstate oxygen equivalent to the scissile oxygen in the substrate. The tungstate bound structure (residue 354 to 358) and the unbound structure are shown in dark and light, respectively.

the Asp residue (general acid) close to the scissile oxygen of the substrate to effect catalysis. Following the chemical step, the loop has to reopen to allow the release of the phosphate product. This apparent ligand-induced loop closure is likely to be operative for the entire PTPase family, although this has not been confirmed for the dual specificity phosphatases and the low-molecular-weight phosphatases, as only the ligand-bound structures have been determined.

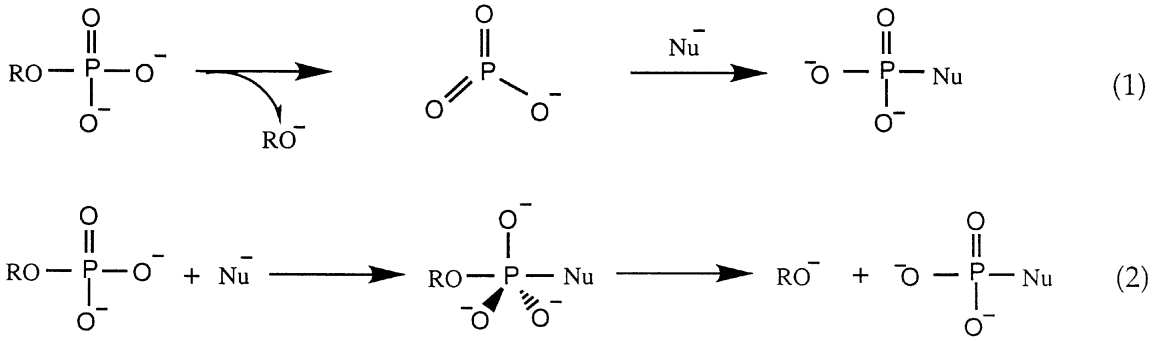
In sum, the core active site structural elements common to the PTPases, the dual

specificity phosphatases, and the low-molecular-weight phosphatases are the PTP loop and the adjacent surface loop that contains the essential Asp residue. Superposition of the active site structures shows that the spatial arrangement of the PTP loop and the moveable loop are conserved (Figure 5 and Fauman and Saper, 1996). This suggests that phosphatases with the PTPase signature motif are likely to recognize the phosphoryl group in a similar way and have similar catalytic mechanisms. These phosphatase structures, taken together with biochemical and mutational studies of active site residues, provide a unique opportunity to identify common mechanistic features associated with this novel family of biological catalysts. In addition, the availability of the structures combined with the ability to mutate specific residues provide an opportunity to test novel mechanistic hypotheses and identify key residues for catalysis and substrate/inhibitor recognition.

V. MECHANISM OF CATALYSIS

A. Nonenzymatic Hydrolysis of Phosphate Monoesters

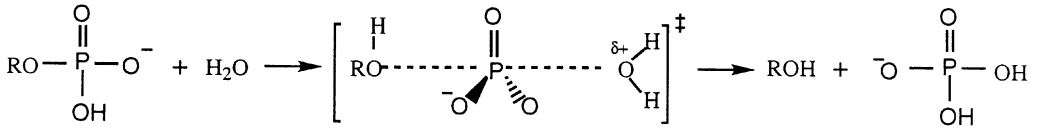
Phosphate monoester hydrolysis is an extremely important reaction in the biological world. It is coupled with energy production, regulation of metabolism, and signal transduction pathways. The mechanism of phosphate monoester hydrolysis has been the subject of intense investigations for over 4 decades (Jencks, 1962; Bruice and Benkovic, 1966; Benkovic and Schray, 1978; Knowles, 1980; Westheimer, 1987; Cullis, 1987; Frey, 1989; Thatcher and Kluger, 1989; and Cleland and Hengge, 1995). Phosphoryl transfer reaction can, in principle, occur via one of two limiting mechanisms analogous to those for substitution at tetrahedral sp^3 carbon (Cox and Ramsay, 1964, Benkovic and Schray, 1978): dissociative (SN1-like, Eq. 1) or associative (SN2-like, Eq. 2). In the SN1 mode, the departure of the leaving group proceeds the nucleophilic attack and there is a decrease of electron density on the phosphoryl group. Dissociation of the leaving group produces a highly electron deficient trigonal phosphorus species, "monomeric metaphosphate", as an intermediate (Butcher and Westheimer, 1955; Westheimer, 1981). In the SN2 mode, the nucleophilic attack proceeds leaving group departure and there is an increase of electron density on the phosphoryl group. In this case, phosphorus compounds have the further potential compared with carbon compounds of reacting via a stable trigonal bipyramidal



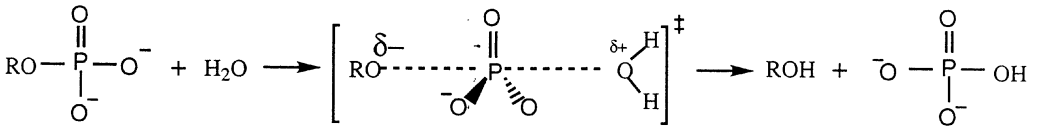
phosphorane intermediate (Benkovic and Schray, 1978). In reality, the metaphosphate and phosphorane intermediates represent only two extremes of a full continuum of possible transition states for phosphoryl transfer reactions.

There is much evidence to support the proposal that the monoanion and dianion of phosphate monoesters hydrolyze via a dissociative mechanism. For example, the hydrolysis of aryl phosphate dianions displays a β_{lg} value of –1.23 (Kirby and Varvoglis, 1967). The Brønsted β_{lg} value is obtained as the slope of the logarithm of rate for a reaction vs. the leaving group pK_a values. The β_{lg} value can often be regarded as an approximate measure of the extent of bond cleavage between the reaction center and the leaving group. Thus, the β_{lg} value of –1.23 for the dianion reaction suggests that in the transition state the P-O bond breaking is well advanced and the mechanism is presumably a simple elimination. The rate of phosphate monoester hydrolysis shows a maximum at pH 4, where the concentration of the monoanion is highest. The β_{lg} value for the monoanion hydrlysis is –0.27, which indicates a unimolecular decomposition with either concerted or preequilibrium protonation of the leaving group (Kirby and Warren, 1967). Results from ^{15}N kinetic isotope studies of the hydrolysis *p*-nitrophenyl phosphate are consistent with a concerted mechanism, although the proton transfer lags slightly behind the P-O bond cleavage (Hengge et al., 1994). The sizable ^{18}O primary kinetic isotope effects on the bridge oxygen also indicate a large degree of P-O bond breakage in the transition state (Gorenstein et al., 1977; Hengge et al., 1994). The entropies of activation for the hydrolysis of phosphate monoesters are close to zero or slightly positive, which is commonly observed in unimolecular decompositions. In contrast, the entropies of activation for bimolecular reactions are typically around –20 e.u. (Kirby and Varvoglis, 1967). The marked increase in the rate of 2,4-dinitrophenyl phosphate by organic solvent is in accord with a transition state in which charge is dispersed (Kirby and Varvoglis, 1967). Secondary ^{18}O kinetic isotope effect in the nonbridging positions of the phosphate group (Weiss et al., 1986; Cleland and Hengge, 1995) is consistent with a dissociative transition state for nonenzymatic hydrolysis of phosphate monoesters. Finally, reactions of phosphate monoesters show very small sensitivity to the basicity of the attacking nucleophilic reagent, displaying $\beta_{nu:}$ value in the range of 0.1 to 0.2 (Jencks and Gilchrist, 1964; Jencks and Gilchrist, 1965; Kirby and Jencks, 1965; Herschlag and Jencks, 1989; Admiraal and Herschlag, 1995). The Brønsted $\beta_{nu:}$ value is obtained as the slope of the logarithm of the rate for a reaction vs. the pK_a values of the attacking nucleophiles. The $\beta_{nu:}$ value can often be viewed as an approximate estimate of the extent of bond formation between the nucleophile and the reaction center. These small $\beta_{nu:}$ values suggest that the reaction is concerted with a small amount of bond formation between the phosphorus and the nucleophile in the transition state. Thus, all of the available data in aqueous solution can be interpreted by a concerted SN2 mechanism involving an unsymmetrically "exploded" metaphosphate-like transition state where bond formation to the incoming nucleophile is minimal and bond breaking between phosphorus and the leaving group is substantial (Bourne and Williams, 1984; Skoog and Jencks, 1984; Jencks et al., 1986; Herschlag and Jencks, 1986; Herschlag and Jencks, 1989). The transition states for the monoanion and dianion phosphate monoester hydrolysis reactions are dipicted in Equations 3 and 4, respectively. There is no direct evidence for the existence of a free metaphosphate intermediate in aqueous solutions (Jencks, 1980; Herschlag and Jencks,

1986). A free metaphosphate has only been implicated in neat solvent such as *t*-butanol (Friedman et al., 1988).



(3)



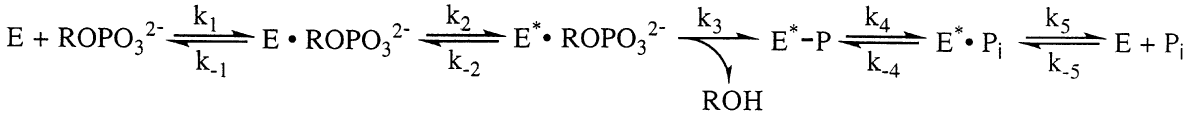
(4)

B. PTPase-Catalyzed Hydrolysis of Phosphate Monoesters

1. The Cys Residue in the PTP-Loop

Enzymological and structural studies have led to the conclusion that the reactions catalyzed by the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases share a common chemical mechanism (Zhang, 1997). Site-directed mutagenesis experiments show that the Cys residue in the PTP loop (e.g., Cys403 in the *Yersinia* PTPase, Cys215 in PTP1, Cys 124 in VHR, and Cys12 in the bovine low-molecular-weight phosphatase) is required for phosphatase activity (Streuli et al., 1990; Guan and Dixon, 1990; Gautier et al., 1991; Cirri et al., 1993; Zhou et al., 1994). The Cys residue is phosphorylated through a thiophosphate linkage ($^{-}S^{-}PO_3^{2-}$) during catalytic turnover (Zhang, 1990; Guan and Dixon, 1991; Wo et al., 1992; Cho et al., 1992; Denu et al., 1996), suggesting a double displacement mechanism, in which the phosphoryl group is first transferred to the thiol group of the Cys residue forming a phosphoenzyme intermediate, which in a second step is hydrolyzed by water. Thus, the minimal kinetic scheme for the PTPase-catalyzed hydrolysis can be represented by Scheme 1. Substrate ($ROPO_3^{2-}$) binding leads to the formation of an enzyme-substrate complex ($E \cdot ROPO_3^{2-}$), which is followed by a binding stabilized conformational change in the enzyme that brings the general acid close to the scissile oxygen of the substrate ($E^* \cdot ROPO_3^{2-}$). Substrate is then cleaved with the departure of the leaving group (ROH) and the phosphoryl transfer to the nucleophilic Cys residue forming a covalent phosphoenzyme intermediate (E^*-P), which is then hydrolyzed by water generating the non-covalent enzyme phosphate complex ($E^* \cdot P_i$). Dissociation of the inorganic phosphate from the enzyme completes the catalytic cycle.

Scheme 1



Based on the crystal structures discussed above, the thiolate anion of the Cys residue is situated at the base of the active site in position for nucleophilic attack on the phosphate ester. The formation and decay of the intermediate (E^{*}-P) has been shown to be kinetically competent by fast chemical quench technique (Cho et al., 1992). Thus, the involvement of a phosphocysteine intermediate in a phosphatase-catalyzed reaction is novel. In addition to the phosphatases, the only other occurrence of a thiophosphate linkage in enzyme systems has been reported in the bacterial sugar carrier-specific transporter enzyme IIB (Pas et al., 1991; Gemmecker et al., 1997). Interestingly, the crystal structure of the IIB enzyme specific for cellobiose show that the fold of $IIB^{cellobiose}$ is remarkably similar to that of the low-molecular-weight phosphatases (van Montfort et al., 1997). Previous work suggests that nonenzymatic thiophosphate ester hydrolysis exhibits many of the characteristics as their oxygen counterparts (Herr and Koshland, 1957; Dittmer et al., 1963; Bruice and Benkovic, 1966; Milstien and Fife, 1967). Because the bond energy of P-S bond (45 to 50 kcal/mol) is considerably less than P-O bond energy (95 to 100 kcal/mol), P-S bond cleavage is much more facile than P-O cleavage (Bruice and Benkovic, 1966). Thus, it is facile that these phosphatases utilize a thiophosphate as a covalent enzyme intermediate in catalysis.

2. The Arg Residue in the PTP Loop

Mutations of the invariant Arg residue in the PTPase signature motif resulted in loss of enzymatic activity for two receptor-like PTPases, LAR and CD45 (Streuli et al., 1990; Johnson et al., 1992), and the low-molecular-weight phosphatase from bovine liver (Cirri et al., 1993). It is not clear, however, from such studies whether the Arg residue is essential for PTPase folding and structure or catalysis, or both. The extraordinary high intrinsic activity of the *Yersinia* PTPase (Zhang et al., 1992) has made it possible to examine the effect of altering the invariant Arg residue within the active site (Zhang et al., 1994b). A 8200-fold decrease in k_{cat} and a 26-fold increase in K_m was observed for the R409A mutant. Interestingly, the R409K mutant displayed a k_{cat} value identical to that of R409A, and the apparent K_m value for *p*-nitrophenyl phosphate was only 1.9-fold higher than that of the wild-type enzyme. Similar results were also obtained for PTP1B (Flint et al., 1997). Using the tyrosine phosphorylated, reduced, carboxamidomethylated, and maleylated lysozyme as a substrate, the K_m for the R221M mutant PTP1B increased by 10-fold, whereas the k_{cat} value decreased by 18,700-fold. The R221K mutant bound the substrate normally, whereas the k_{cat} value was reduced by 5500-fold. These results suggest that although the Arg residue in the PTP loop plays a role in substrate binding, it plays a much more important role in transition state stabilization.

The guanidinium group (present in an arginine residue) is ideally suited for interaction with a phosphate group by virtue of its planar structure and its ability to form multiple hydrogen bonds with the phosphate moiety (Cotton et al., 1973). The three-dimensional structures of the PTPase-oxyanion complexes show that two of the oxyanion oxygens ion pair with the positively charged guanidinium group of the Arg residue. This explains the requirement for the Arg residue in substrate binding. The ability of the guanidinium group to form a coplanar bidentate complex with two of the equatorial oxygen atoms present on the phosphate during catalysis provides a plausible mecha-

nism for stabilization of the trigonal bipyramidal transition state(s). The geometry associated with the amino group of the alternate cationic Lys side chain would not be expected to be able to form a coplanar bidentate complex with the trigonal bipyramidal transition state, which may explain why the Arg to Lys mutant showed little improvement in k_{cat} when compared with the Arg to Ala or Met mutants. The fact that a Lys residue can partially replace the Arg residue for substrate binding but fails in assisting catalysis suggests that the transition state(s) likely employ the unique structural properties of the guanidinium side chain of the arginine. It is most likely that the conserved Arg residue in the PTPase active site is geometrically positioned in such a manner that it interacts more favorable with the transition state than the ground state.

3. The Conserved Ser/Thr in the PTP Loop

In addition to the essential Cys and Arg residues, a conserved Ser or Thr can also be found in the PTPase signature motif immediately after the invariant Arg residue (Figure 3, Zhang et al., 1995b). The dual specificity phosphatase cdc25 is the only exception that lacks a hydroxyl group at this position. Interestingly, cdc25 is several orders of magnitude less reactive than other PTPases (Dumphy and Kumagai, 1991; Zhang et al., 1992). In the bovine low-molecular-weight phosphatase structure (Su et al., 1994; Zhang, M. et al., 1994), the VHR structure (Yuvaniyama et al., 1996), as well as in the *Yersinia* PTPase (Stuckey et al., 1994) and the human PTP1B (Barford et al., 1994) structures, the hydroxyl group of the conserved Ser/Thr is approximately 3 Å to the Sγ of the active site Cys residue, making a reasonable good S-HO hydrogen bond (Gregoret et al., 1991). Eliminating this hydrogen bond could have an effect on the stability of the thiolate ion and on the phosphatase-catalyzed reaction.

To illuminate the function of the conserved hydroxyl group, the Ser/Thr to Ala mutants of the PTPases (Zhang et al., 1995b; Lohse et al., 1997), the dual specificity phosphatase VHR (Denu and Dixon, 1995) and the yeast *Schizosaccharomyces pombe* low-molecular-weight phosphatase Stp1 (Zhao and Zhang, 1996) were prepared and analyzed by pre-steady state stopped-flow kinetic experiments. Elimination of the conserved hydroxyl group in the PTP loop has only a modest effect on k_3 (E^*-P formation), whereas its major impact seems to be primarily reflected in the dramatic decrease in k_4 (E^*-P decomposition). For example, in the reaction catalyzed by the *Yersinia* PTPase, elimination of the hydroxyl group at Thr410 decreases the rate of E^*-P formation (k_3) and breakdown (k_4) by 2.4- and 30.4-fold, respectively (Zhang et al., 1995b). In VHR, the Ser 131 to Ala mutation leads to 3-fold drop in k_3 and more than 100-fold drop in k_4 (Denu and Dixon, 1995). Similarly, substitution of the corresponding Ser18 by an Ala in Stp1 resulted in a 4.3- and 35.7-fold reduction in k_3 and k_4, respectively (Zhao and Zhang, 1996). In contrast, when the S19A mutant of the bovine low-molecular-weight phosphatase was examined by steady-state kinetic techniques, it was found that the hydroxyl group played a critical role in E^*-P formation and that its removal resulted a change in the rate-limiting step from E^*-P hydrolysis in the wild-type to E^*-P formation in the mutant (Evans et al., 1996). The mammalian and the yeast low-molecular-weight phosphatases share 42% amino acid sequence identity (Mondesert et al., 1994). It is not clear what causes this apparent discrepancy and further investigation is required. Thus, results from the

Yersinia PTPase, PTP1, the dual specificity phosphatase VHR, and the low-molecular-weight phosphatase Stp1 suggest that the main function for the conserved hydroxyl group in the PTPase signature motif is to facilitate the breakdown of the phosphoenzyme intermediate. The differential effects on the two chemical steps by the elimination of the hydroxyl-thiolate interaction is discussed in light of the nature of the transition state in the phosphatase-catalyzed reaction.

4. General Acid/Base Catalysis

In addition to nucleophilic catalysis and transition state stabilization, it appears that all three groups of phosphatases also utilize general acid/base to facilitate the catalytic turnover. Based on the observation that substitution of the invariant Asp356 in the *Yersinia* PTPase to Asn reduces catalytic activity by three orders of magnitude and that Asp356 is responsible for the basic limb of the pH rate profile, it has been proposed that Asp356 acts as a general acid in the reaction mechanism (Zhang et al., 1994a). Subsequently, the involvement of an aspartic acid as a general acid has also been established in the low-molecular-weight phosphatases (Asp129, Zhang, Z. et al., 1994; Taddei et al., 1994; Wu and Zhang, 1996), in the dual specificity phosphatase VHR (Asp92, Denu et al., 1995), and in the mammalian PTP1-catalyzed reaction (Asp181, Hengge et al., 1995; Zhang, 1997; Lohse et al., 1997).

The proposed involvement of general acid/base catalysis in reactions of the PTPase, the dual specificity phosphatases, and the low-molecular-weight phosphatases is supported by structural studies. The three-dimensional structures of the phosphatase-oxyanion complexes reveal that the carboxylate of Asp356 in the *Yersinia* PTPase or its structural counterparts in VHR and low-molecular-weight phosphatase are in hydrogen bonding distance from the apical oxygen of the bound oxyanion (Stuckey et al., 1994; Su et al., 1994; Zhang, M. et al., 1994; Yuvaniyama et al., 1996). Furthermore, the crystal structures of the *Yersinia* PTPase and the bovine low-molecular-weight phosphatase complexed with the transition state analog vanadate have also been solved (Denu et al., 1996; Zhang, M. et al., 1997). Vanadate ion forms a covalent bond with the active site Cys residue and exhibits a trigonal bipyramidal geometry. The carboxylate of Asp356 in the *Yersinia* PTPase and Asp129 in the low-molecular-weight phosphatase makes a hydrogen bond to the apical oxygen of vanadate, consistent with its role as a general acid/base. The apical oxygen is structurally homologous to the scissile oxygen of a phosphate monoester substrate in the Michaelis complex ($E^{*}{\cdot}ROPO_3^{2-}$) or the attacking water molecule in the E^{*}-P complex. Thus, it is highly likely that during the E^{*}-P formation step, the aspartic acid acts as a general acid to donate a proton to the phenolic/alcoholic oxygen, facilitating the expulsion of the leaving group. This is in accord with the observation that in the structure of PTP1B/C215S bound with a pTyr-containing peptide, the phenolic oxygen of pTyr forms a network of hydrogen bonds with the side chain of the corresponding Asp181 and a buried water molecule (Jia et al., 1995). After the formation of the E^{*}-P, the dephosphorylation event would occur by attack of a water molecule that approaches from the just-vacated leaving group side on the E^{*}-P with subsequent release of inorganic phosphate. It is thus conceivable that the Asp residue could function in the second step by activating a water molecule for the hydrolysis of the E^{*}-P.

Although the importance of an Asp residue in catalysis has been confirmed by both biochemical and structural data, there were disagreements about the detailed mechanism and the specific step(s) that is effected by the Asp residue (Taddei et al., 1994; Zhang, Z. et al., 1994). In addition, the necessity and/or the identity of a general base in E^*-P hydrolysis have also been questioned (Zhang, M. et al., 1994; Fauman et al., 1996). The mechanism and the specific step(s) that is effected by the Asp residue has been revealed by a detailed investigation that utilizes a combination of techniques, including site-directed mutagenesis, pre-steady-state and steady-state kinetic analysis (Wu and Zhang, 1996). Asp128 in the yeast enzyme Stp1 has been replaced by a Glu, an Asn, and an Ala. The k_{cat} for the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) decreases by factors of 6.7, 400, and 650 for the mutants D128E, D128N, and D128A. An evaluation of the burst kinetics demonstrates that Asp128 plays a role in both the E^*-P formation (k_3) and breakdown (k_4) (Figure 8). Thus, substitution at Asp128 by a Glu, an Asn, or an Ala reduces k_3 by 17, 7480, and 11900-fold and k_4 by 6.2-, 380-, and 40-fold. The greater effect on k_3 than k_4 is consistent with a dissociative transition state for the low-molecular-weight phosphatase-catalyzed reaction (see below). Taken together, these results are consistent with that Asp128 acts as a general acid to donate a proton to the phenolate leaving group in the phosphorylation step (k_3), and the same carboxylate side chain plays a role as a general base to activate a nucleophilic water molecule in the dephosphorylation step (k_4). Furthermore, kinetic analysis of a double mutant phosphatase, D181N/S222A in PTP1 (Lohse et al., 1997), and D92N/S131A in VHR (Denu et al., 1996), also demonstrated the involvement of the Asp in the E^*-P hydrolysis step in PTP1 and VHR catalyzed reaction.

5. The Nature of the Transition States

As discussed above, nonenzymatic nucleophilic displacement reactions on phosphate monoesters are believed to involve an "exploded" metaphosphate-like dissociative transition state. A prerequisite for the detailed understanding of PTPase-catalyzed phosphoryl transfer reactions is the elucidation of the nature of the enzymic transition state. The catalytic power of the *Yersinia* PTPase is illustrated by the 10^{11}-fold rate acceleration of *p*NPP hydrolysis over the dianion solution reaction (Zhang et al., 1994a). Given that the phosphomonoester dianion reactions exhibit a β_{lg} of –1.23, and that the *Yersinia* PTPase-catalyzed reaction displays little leaving group dependency (Zhang et al., 1994c), the rate enhancement for phosphotyrosine hydrolysis by the *Yersinia* PTPase could be over 10^{14}. What is the preferred mechanistic route for a PTPase-catalyzed phosphoryl transfer reactions? Do PTPases catalyze phosphoryl transfers via a dissociative or an associative mechanism? Different strategies may be utilized by a PTPase to accelerate a reaction depending on the nature of the transition state.

An associative mechanism had been favored for enzyme-catalyzed phosphoryl transfer reactions, as it is easy to imagine that an enzyme could stabilize the increased negative charge on the phosphoryl moiety in the transition state (Hasset et al., 1982; Mildvan and Fry, 1987; Cullis, 1987). It has been argued that general acid catalysis to accelerate the departure of leaving group should be enzymatically important regardless of the mechanism of phosphorylation (i.e., associative or dissociative) and that there should be little requirement for general base catalysis in dissociative mechanism (Benkovic and Schray, 1978). In order to maximize the catalytic power from proton transfer to a general base at the active

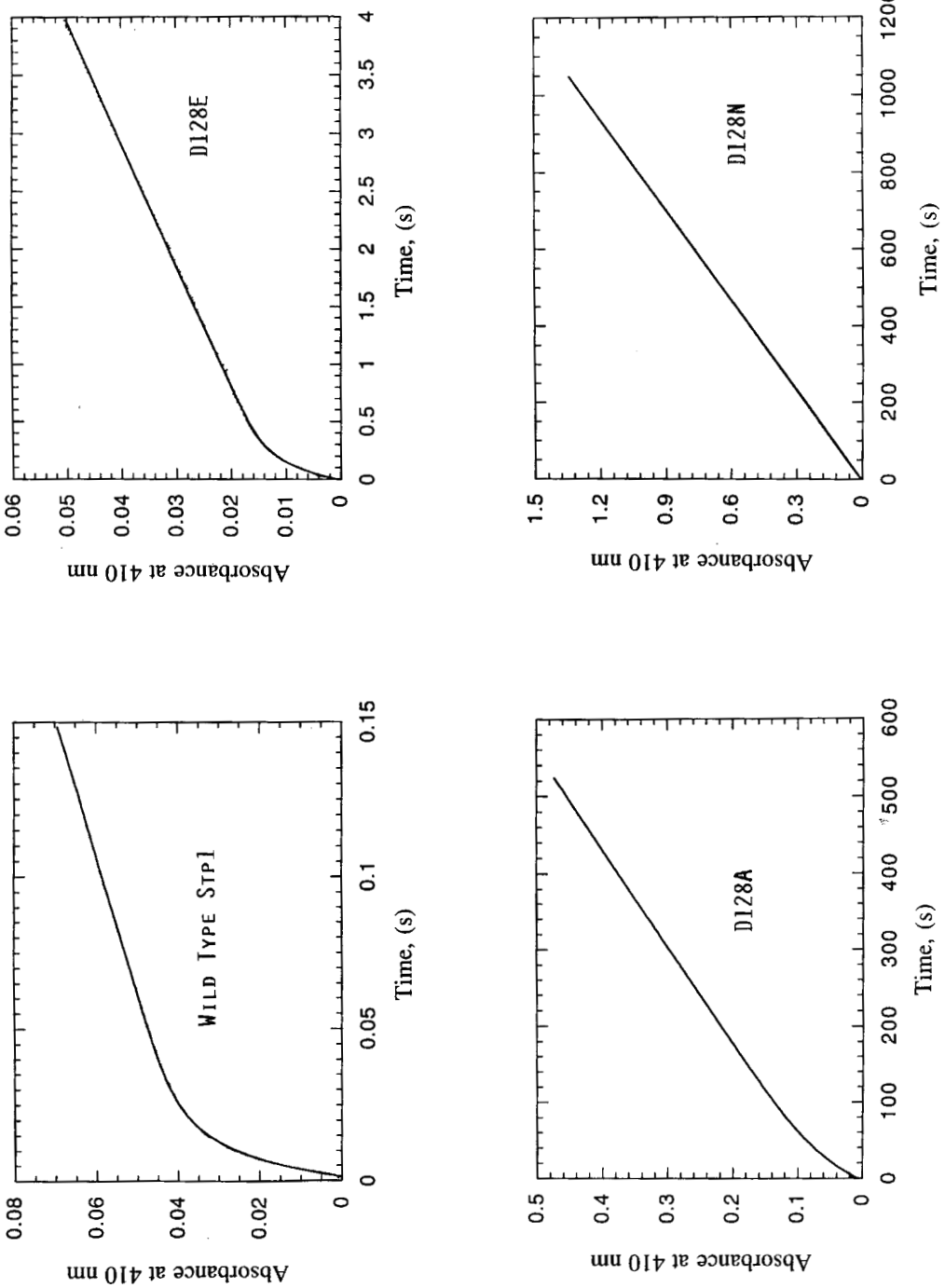


FIGURE 8. Burst kinetics observed with Stp1 and *p*NPP at pH 6 and 30°C. (From Wu and Zhang, 1996.)

site, it is conceivable that the enzyme may select a somewhat more associative transition state with more bond formation to the entering nucleophile (Herschlag and Jencks, 1989).

a. Transition State for the E-P Formation Step*

Heavy-atom kinetic isotope effects have been measured in order to determine the nature of the transition state for the reaction catalyzed by the PTPases (Hengge et al., 1995), the dual specificity phosphatases (Hengge et al., 1996), and the low-molecular-weight phosphatases (Hengge et al., 1997). Isotope effects on three sites in *p*NPP (Figure 9) were measured by the competitive method using an isotope ratio mass spectrometer. The competitive method (O'Leary and Marlier, 1979) measures effects on k_{cat}/K_m, which is sensitive only to the early part of the mechanism through the first irreversible step, shown as k_3 in Scheme 1. The secondary ^{18}O isotope effect in the nonbridge phosphoryl oxygen atoms in the substrate, $^{18}(V/K)_{nonbridge}$, determines whether the transition state is dissociative (metaphosphate-like) or associative, resembling a pentavalent phosphorane (Cleland and Hengge, 1995). The primary ^{18}O isotope effect in the bridge oxygen atom in the substrate, $^{18}(V/K)_{bridge}$, gives an indication of the extent of P-O bond cleavage. The secondary ^{15}N isotope effect in the nitrogen atom of the leaving group, $^{15}(V/K)$, is sensitive to the amount of negative charge delocalized into the aromatic *p*-nitrophenol ring and gives a measure of the charge that is developed on the phenolic oxygen in the transition state (Hengge et al., 1994). Table 1 summarizes the isotope effects for the phosphatase-catalyzed *p*NPP reactions. For comparison, the isotope effects for the nonenzymatic solution reactions of *p*NPP are listed in Table 2. The fact that the isotope effects on the enzymatic reactions are comparable with those of the solution reactions suggests that chemistry, that is, P-O bond cleavage, is rate-limiting for the k_{cat}/K_m portion of the mechanism and that substrate binding and the putative binding-induced conformational change are both rapidly reversible and do not introduce a commitment toward catalysis.

The $^{18}(V/K)_{nonbridge}$ isotope effects of the phosphatase reaction should be compared with those of the dianion solution reactions because the phosphatase utilize the dianion form of the substrate for effective catalysis

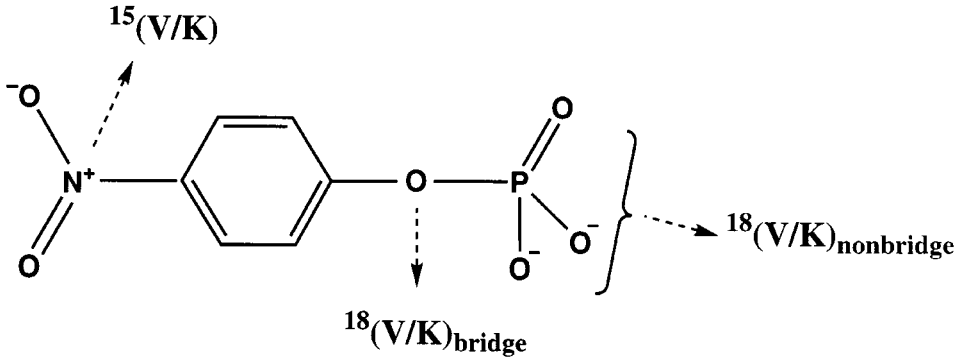


FIGURE 9. Heavy atom kinetic isotope effects measured at three different positions in *p*NPP.

TABLE 1
Kinetic Isotope Effects on Reactions of *Yersinia* PTPase, PTP1, VHR, and Stp1 with *p*NPP[a]

Phosphatase	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
Yersinia PTPase[b]			
WT	0.9999 ± 0.0003	1.0160 ± 0.0015	1.0001 ± 0.0013
D356N	1.0024 ± 0.0005	1.0275 ± 0.0016	1.0022 ± 0.0005
D356A	1.0022 ± 0.0003	1.0274 ± 0.0008	1.0007 ± 0.0005
PTP1[b]			
WT	1.0002 ± 0.0004	1.0170 ± 0.0020	0.9981 ± 0.0015[c]
D181N	1.0019 ± 0.0002	1.0278 ± 0.0017	1.0018 ± 0.0003
VHR[d]			
WT	0.9999 ± 0.0004	1.0118 ± 0.0020	1.0003 ± 0.0003
D92N	1.0030 ± 0.0002[e]	1.0294 ± 0.0009[e]	1.0019 ± 0.0005[e]
S131A	1.0002 ± 0.0003[e]	1.0119 ± 0.0005[e]	1.0001 ± 0.0006[e]
Stp1[f]			
WT	1.0007 ± 0.0001	1.0160 ± 0.0005	1.0018 ± 0.0003
D128N	1.0034 ± 0.0003	1.0282 ± 0.0012	1.0024 ± 0.0005
D128A	1.0030 ± 0.0005	1.0297 ± 0.0009	1.0010 ± 0.0003
D128E	1.0006 ± 0.0002	1.0166 ± 0.0010	1.0013 ± 0.0003
D18A	1.0010 ± 0.0002	1.0172 ± 0.0013	1.0024 ± 0.0005

[a] All measurements were made at pH 6 unless otherwise indicated.
[b] Hengge et al., 1995.
[c] Measured at pH 5.5.
[d] Hengge et al., 1996.
[e] Measured at pH 7.
[f] Hengge et al., 1997.

TABLE 2
Kinetic Isotope Effects on the Uncatalyzed Solution Reactions of *p*NPP[a]

*p*NPP	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
Monoanion	1.0005 ± 0.0002	1.0106 ± 0.0003	1.0224 ± 0.0005
Dianion	1.0034 ± 0.0002	1.0230 ± 0.0005	0.9993 ± 0.0007
Dianion in *tert*-butanol	1.0039 ± 0.0003	1.0202 ± 0.0008	0.9997 ± 0.0016

[a] Hengge et al., 1994.

(Zhang et al., 1994c; Zhang, 1995; Denu et al., 1995; Hengge et al., 1997). The $^{18}(V/K)_{nonbridge}$ isotope effect is a predictable and valuable tool in assessing the dissociative vs. associative character of a transferring phosphoryl group in the transition state (Cleland and Hengge, 1995), because its value is negligible or slightly inverse for dissociative mechanisms typical of monoesters, small but normal (typically from 1.003 to 1.006) for diesters of *p*-nitrophenol where the transition state is somewhat associative, larger, and normal (1.006 to 1.025) for triesters where the reaction is more associative (Caldwell et al., 1991). The near unity or slightly inverse values for the *Yersinia* PTPase, PTP1, and VHR are similar to those of solution reactions of dianion and is consistent with the known metaphosphate-like nature of the phosphoryl group in

the dissociative transition state. Interestingly, the magnitude of the $^{18}(V/K)_{nonbridge}$ effect for the Stp1 reaction borders between those of the dianion reactions in solution and the diesters of *p*-nitrophenol, suggesting that the transition state for the Stp1 reaction is intermediate in structure between the metaphosphate-like monoester reaction and the somewhat associative nature found in diester reaction. Thus, there appears to be a greater nucleophilic interaction between the Cys nucleophile and phosphoryl group causing some decrease in nonbridge P-O bond order in the Stp1 reaction. It is also interesting to note that in the general acid-deficient mutant PTPases and dual specificity phosphatases (D356N in *Yersinia* PTPase, D181N in PTP1, and D92N in VHR) the $^{18}(V/K)_{nonbridge}$ values are small and normal, which is consistent with the need for greater nucleophilic involvement in the absence of general acid assistance. Thus, the central phosphoryl group in the transition state of the mutant phosphatase catalyzed reactions is less metaphosphate-like than the wild-type enzyme.

The magnitude of ^{15}N isotope effect also varies in a systematic fashion as expected in reactions of monoesters, diesters, and triesters of *p*-nitrophenol (Hengge and Cleland, 1990). This isotope effect is largest in the aqueous hydrolysis of the dianion where essentially a full negative charge is developed on the leaving group in the transition state. The magnitude of this ^{15}N isotope effect decreases in diester reactions and decreases further in reactions of triesters. In this series the transition states become more associative and the P-O bond cleavage less advanced. The $^{15}(V/K)$ effects for the *Yersinia* PTPase, PTP1, and VHR are effectively unity, indicating complete charge neutralization due to proton transfer from the general acid in the transition state. In contrast, the small but experimentally significant value of 1.0007 for $^{15}(V/K)$ with the native Stp1 and 1.0005 for the aqueous hydrolysis of the monoanion indicate that in these cases proton transfer to the leaving group lags slightly behind P-O bond cleavage, resulting in the leaving group bearing a small amount of negative charge in the transition state. With the elimination of the proton donor in the phosphatases, the leaving group departs as the *p*-nitrophenolate anion. Indeed, the $^{15}(V/K)$ values for the general acid-deficient mutant VHR and Stp1 are similar to those of the dianion reactions, consistent with complete charge delocalization into the ring in the transition state. Interestingly, the $^{15}(V/K)$ values for the general acid-deficient mutant *Yersinia* PTPase and PTP1 are measurably smaller than those of the dianion reactions, suggesting that the general acid may not be the only means for charge stabilization and additional mechanism(s) may exist for PTPases to coneutralize the developing negative charge in the leaving group.

The $^{18}(V/K)_{bridge}$ isotope effects of the phosphatase reaction should be compared with those of the monoanion solution reactions because in both cases the leaving group is protonated and expelled as the neutral species, in contrast to the dianion reaction where it leaves as the *p*-nitrophenolate anion. Thus, the primary isotope effect for the bridge oxygen in monoanion is smaller than that in the solution reactions of dianion, which is due to the fact that the cleavage of the P-O bond is partially compensated for by the transfer of the proton to the phenolic oxygen. The magnitude of the primary ^{18}O isotope effect at the bridge position gives information about the degree of P-O bond cleavage in the transition state. In an associative transition state P-O bond cleavage is less advanced, and its contribution to the size of the isotope effect is therefore less. For example, an $^{18}O_{bridge}$ of 1.0060 was observed for the alkaline hydrolysis of diethyl *p*-nitrophenyl phosphate triester (Caldwell

et al., 1991) that exhibited an associative transition state. Protonation of the leaving group would reduce this value further. The $^{18}(V/K)_{bridge}$ isotope effects of the phosphatases are slightly larger than that of the monoanion reaction and indicate that the degree of P-O bond cleavage in the transition state is advanced. When the general acid is removed, the $^{18}(V/K)_{bridge}$ isotope effects increase in magnitude and are similar to those of the dianion reactions, confirming the high degree of P-O bond cleavage. This supports the proposed role of the conserved Asp residue as a general acid. Collectively, the magnitudes of the $^{18}(V/K)_{bridge}$ isotope effects are also consistent with a dissociative transition state for the phosphatase reaction.

In summary, comparisons of the heavy atom kinetic isotope effects on the reaction catalyzed by the *Yersinia* PTPase, PTP1, VHR, and Stp1 with the solution data suggest that the transition states for phosphorylation step (k_3) of these phosphatase reactions are highly dissociative in character, as is the case for the nonenzymatic reaction (Hengge et al., 1995, 1996, 1997). A dissociative transition state for the phosphorylation of the phosphatases is dipicted in Figure 10, in which P-O bond to the leaving group is largely broken, proton transfer to the leaving group oxygen is correspondingly advanced such that the departing phenol has no charge, and the central phosphoryl group resembles metaphosphate in structure. It is particularly interesting to point out that although the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases employ similar chemical mechanisms for catalysis, measurable differences exist between the transition states of these phosphatase-catalyzed reactions.

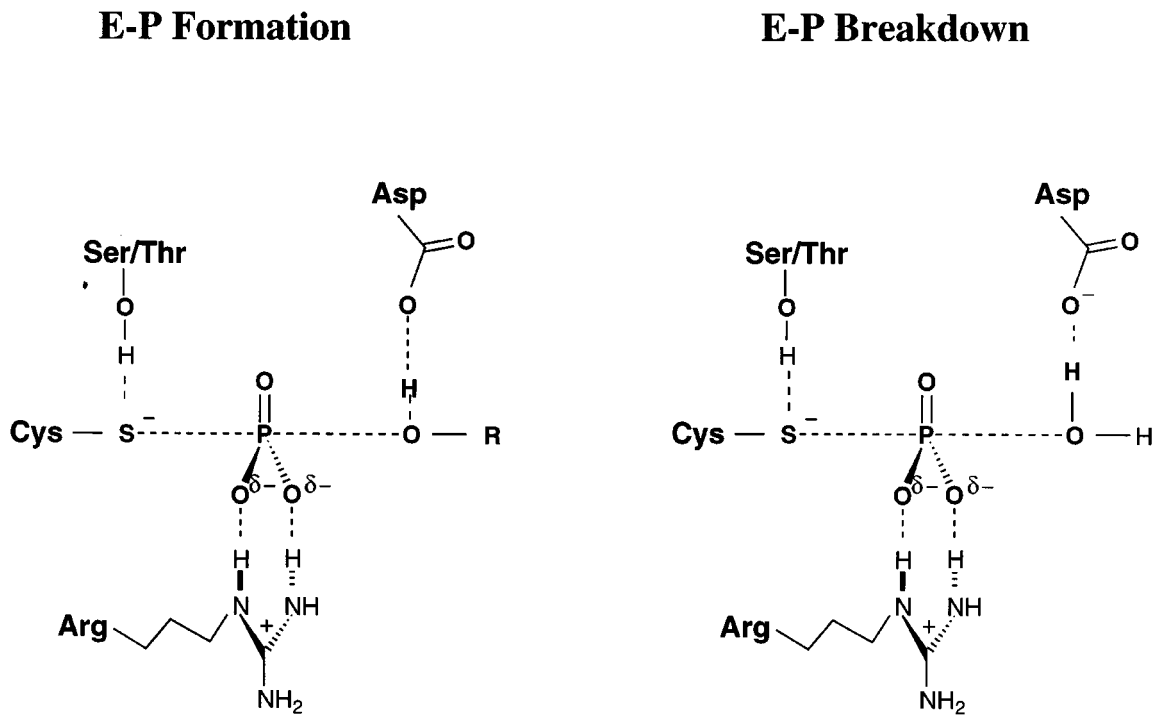
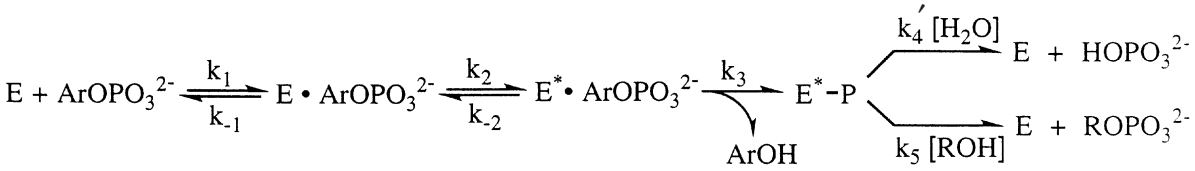


FIGURE 10. Suggested transition state structures for the phosphorylation and dephosphorylation steps in the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases-catalyzed reaction.

Further studies will reveal the structural origins for the differences in transition states.

b. Transition State for the E-P Decay Step*

To probe the transition state of the dephosphorylation step, linear free energy relationship, or Brønsted correlation have been applied to study the effect of changing pK_a on the second-order rate constants for the reaction of β-substituted ethanols with the phosphoenzyme intermediate (E*-P) (Zhao and Zhang, 1996). This is based on the fact that E*-P in the low-molecular-weight phosphatase-catalyzed reaction can partition with water to give the hydrolysis product (inorganic phosphate) and with alcohols to produce the alkyl phosphates (Zhang and Van Etten, 1991a; Wu and Zhang, 1996). Scheme 2 illustrates the partitioning of E*-P in the presence



of alcohol ROH, in which k_4 (= $k_4'[H_2O]$) is the rate of hydrolysis while $k_5[ROH]$ is the rate of E*-P reacting with ROH to form the phosphorylated alcohol ($ROPO_3^{2-}$). The $\beta_{nu:}$ parameter, obtained from structure-reactivity correlations of reactivity as a function of the pK_a of the attacking nucleophile, may be viewed as an empirical index of the fraction of charge transferred to the nucleophile and correspondingly may reflect the degree of bond formation between the nucleophile and the phosphorus in the transition state (Jencks and Gilchrist, 1968). Thus, information about the transition state of E*-P dephosphorylation can be obtained by studying the selective influence of a nucleophilic acceptor on the partitioning of E*-P in the Stp1-catalyzed *p*NPP hydrolysis. The $\beta_{nu:}$ value for the Stp1-catalyzed reaction is 0.14, which falls in the range of 0.1 to 0.2 observed for solution reactions of monoesters. The small dependence of the second-order rate constant (k_5) on basicity for the reactions of alcohols with E*-P suggests that the transition state for the dephosphorylation of E*-P in the Stp1-catalyzed reaction is also highly dissociative, similar to the solution reactions (Figure 10). Furthermore, the fact that there is dependence of phosphoryl transfer on the nucleophile basicity also indicates that the entering nucleophile is a required participant in the E*-P reaction pathway and argues against the existence of a free metaphosphate intermediate in the Stp1-catalyzed reaction. This is particularly interesting, because in solution μ-monothiopyrophosphate is millions of times more reactive than pyrophosphate as a phosphoryl donor to water. In the case of μ-monothiopyrophosphate, the much weaker P-S bond undergoes hydrolysis through a metaphosphate-like intermediate with little nucleophilic participation (Lightcap and Frey, 1992). In normal phosphate monoester and anhydride reactions, phosphoryl transfer to nucleophiles include the nucleophile in the dissociative transition state. It appears that the nature of the transition states for the Stp1-catalyzed reaction are highly dissociative and similar to that in nonenzyme-catalyzed solution reaction for both of the chemical steps (i.e., E*-P formation and

breakdown) in the phosphatase-catalyzed reaction.

c. How Is the Transition State Stabilized by the Phosphatases?

The fact that the transition states for the E^*-P formation and hydrolysis are both dissociative is in accord with results from mutational and kinetic investigations. An important strategy employed by the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases to promote a dissociative mechanism is to stabilize the buildup of negative charge on leaving group. The dissociative transition state for E^*-P formation in the phosphatase reaction is stabilized by the active site Asp residue that facilitates the departure of the leaving phenoxide (Hengge et al., 1995; 1996; 1997; Zhang et al., 1995c; and Wu and Zhang, 1996). For example, it is interesting to note that, in general, substitutions at the conserved Asp residue have a more profound effect on the step leading to the intermediate formation, when compared with its decomposition (Wu and Zhang, 1996). This is consistent with a dissociative transition state: a greater help is needed to facilitate the departure of the leaving group in the phosphoenzyme formation step (where the Asp acts as a general acid) than to activate the nucleophilic water in the phosphoenzyme hydrolysis step (where the Asp acts as a general base). The marginal D_2O solvent isotope effects associated with the E^*-P hydrolysis step (Zhang and Van Etten, 1991b; Zhang et al., 1994c; Zhang, 1995; and Zhang et al., 1995c) are consistent with the nature of the transition state and are similar to that observed for solution reaction (Sabato and Jencks, 1961).

Because the transition state for E^*-P dephosphorylation step is also dissociative, charge stabilization on the leaving thiolate is also important because P-S bond breaking is substantial in the transition state (Zhang et al., 1995b; Zhao and Zhang, 1996). This charge stabilization is effected by a hydrogen bond between the sulfur atom of the active site Cys residue and the conserved hydroxyl group in the PTPase signature motif. The loss of such an interaction would make the phosphocysteine more resistant to breakdown. Thus, a more dramatic decrease in the rate of E^*-P hydrolysis is observed when the hydroxyl group is eliminated (Zhang et al., 1995b; Denu and Dixon, 1995; Zhao and Zhang, 1996; and Lohse et al., 1997). One would predict that the elimination of the active site hydroxyl group would make the dephosphorylation reaction less dissociative. Indeed, the $\beta_{nu:}$ value for the Stp1/S18A-catalyzed reaction is 0.26, which is close to those of reactions of oxyanions with phosphate triesters that range from 0.30 to 0.48, depending on the leaving groups (Khan and Kirby, 1970). As there is very little bond formation between the phosphorus and the attacking nucleophile in a dissociative transition state, minimal activation is required for the nucleophilic attack in the E^*-P formation step. Thus, elimination of the hydroxyl group PTPase signature motif has minimal effects on the rate and the structure of the transition state of the phosphorylation step (Table 1).

It must be pointed out that leaving group stabilization is not the only strategy that members of the PTPase superfamily utilize to lower the transition state energy. The general acid Asp residue only contributes a factor of 10^3, whereas the conserved Ser/Thr residue in the PTP loop contribute a factor of 10^2 in rate enhancement. Other features of the enzyme, such as the active site environment, and the Arg residue in the PTP-loop, also play a role in transition state stabilization. Kinetic studies have suggested that the essential Arg residue in the PTP-

loop is involved in substrate binding and transition state stabilization (Zhang et al., 1994b). The X-ray structural data indicate that the guanidinium group of the Arg residue forms bidentate salt bridges with two of the oxyanion oxygens (Stuckey et al., 1994; Barford et al., 1994; Su et al., 1994; Zhang, M. et al., 1994; and Yuvaniyama et al., 1996). The interaction between arginyl side chains and phosphate groups in phosphoryl transfer mechanisms has been discussed by Knowles (1980). Because compared with the ground state, the peripheral oxygen atoms bear more negative charge in an associative transition state and less negative charge in a dissociative transition state, it is conceivable that interaction between an active site Arg residue with phosphoryl oxygens would inhibit the dissociative pathway but promote the associative process. How could an Arg residue stabilize a dissociative transition state? Biochemical studies have shown that the Arg to Lys mutant retains much of the wild-type enzyme substrate binding capacity but loses its ability to effectively hydrolyze the substrate (Zhang et al., 1994b; Flint et al., 1997). These results indicate that the charge at the Arg position is not sufficient for catalysis and that the coplanar geometry of the guanidinium group must be critical for hydrolysis (Zhang et al., 1994b). As the reaction proceeds from the ground state tetrahedral orthophosphate to the metaphosphate-like transition state, the coplanar geometry of the guanidinium group is ideally positioned to stabilize the sp^2 hybridization of the PO_3 oxygens. In the structure of the low-molecular-weight phosphatase complexed with the transition state analog vanadate, a significant movement of the side chain of the essential Arg residue was observed to ensure proper interactions of the terminal nitrogens with two of the three coplanar oxygens of the vanadate trigonal bypyramid (Zhang, M. et al., 1997). It is possible that the decrease in charge density on the phosphoryl oxygens in a dissociative transition state is more than offset by the optimal geometric alignment of the guanidinium group and the terminal phosphoryl oxygens. A comparison of ^{18}O kinetic isotope effects between the wild-type and the Arg409 mutants may lead to a better understanding of the nature of the interaction between the Arg residue and the phosphoryl oxygens in the transition state.

6. The Common Mechanism for Catalysis

The structure of the PTP loop, including the invariant Cys and Arg residues, the essential Ser/Thr residue, and the surface loop bearing the essential Asp residue have been conserved in the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases. It becomes evident from the above discussion that these three group of phosphatases utilize these similar structural features within their active sites and employ a common strategy for phosphate monoester hydrolysis. The common catalytic strategy (Figure 11) involves the Cys residue acting as the nucleophile to attack the phosphate ester, forming a thiophosphate intermediate and the invariant Arg residue playing a role both in substrate recognition and transition state stabilization. The step leading to cysteinyl-phosphate formation is facilitated by the protonation of the ester oxygen atom in the leaving group, which is accomplished by the conserved Asp residue acting as a general acid. This is required for stabilization of the trigonal bipyramidal transition state toward loss of the oxygen of the leaving group rather than the sulfur of the cysteinyl nucleophile. After the formation of the phosphoenzyme intermediate, the dephosphorylation event would occur by attack of

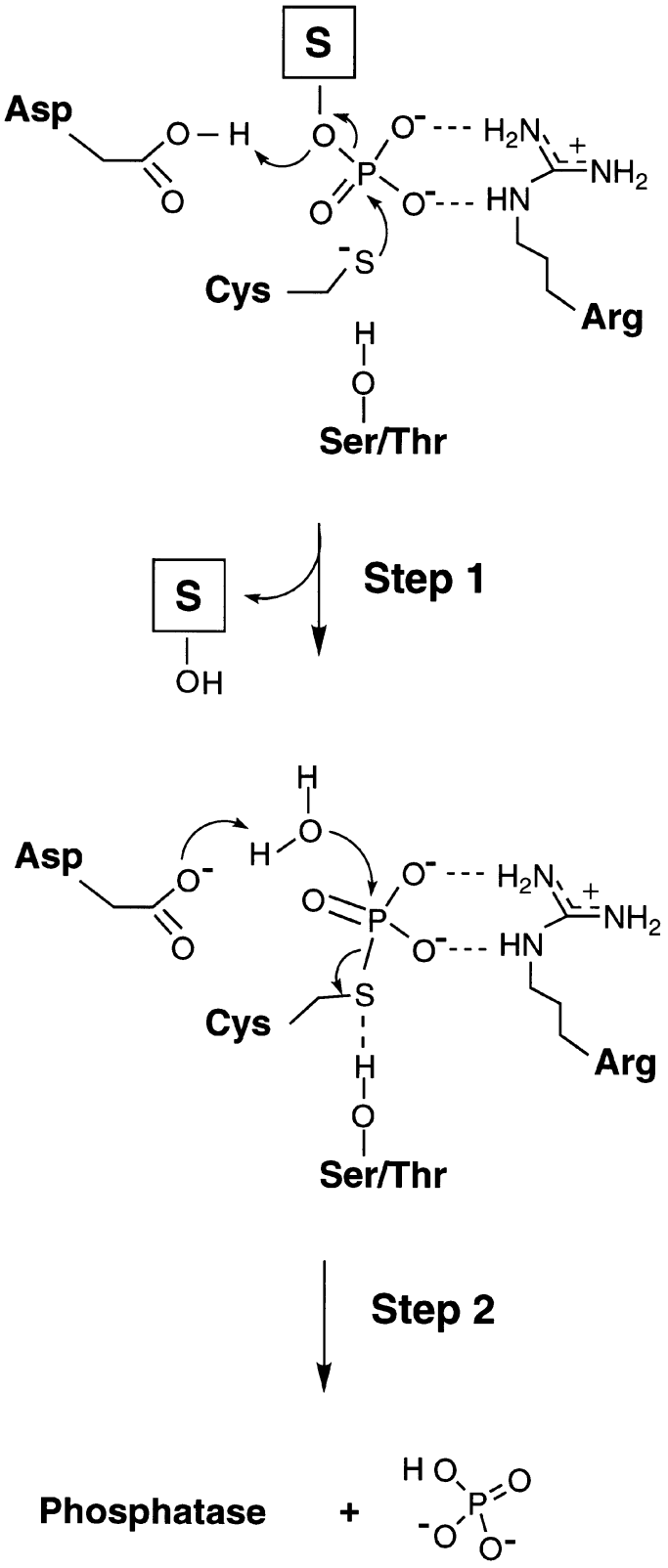


FIGURE 11. The common chemical mechanism for the reaction catalyzed by the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases.

water that approaches from the just-vacated leaving group side on the phosphoenzyme intermediate with subsequent release of inorganic phosphate. The same Asp residue functions as a general base in the second step by activating or positioning a water molecule for the hydrolysis of the phosphoenzyme intermediate. As part of the PTP loop, the conserved Thr/Ser residue immediately following the invariant Arg residue functions to facilitate the breakdown of the phosphoenzyme intermediate. It is particularly interesting that nature appears to have utilized this strategy on more than one occasion to hydrolyze phosphate monoesters (e.g., PTPases and low-molecular-weight phosphatase), and that this strategy differs markedly from those employed by alkaline, acid, or Ser/Thr protein phosphatases.

C. Dynamics of the Movable Loop

Many enzymes are characterized by fluctuating conformations and dynamic motions that are coupled to the binding and release of substrate or product. Such conformational change and motion may function to exclude solvent, recruit essential amino acids for catalysis, or to stabilize and/or prevent loss of reactive intermediates. Although crystallographic studies have documented the extent of the loop motion in various enzymes, the range and frequency of flexible loop motions remain undetermined. The relationship between microscopic protein dynamics and the rate of enzymatic catalysis is unclear. A quantitative description of protein dynamics is important to the mechanistic understanding of enzyme action. Time-resolved spectroscopy have been particularly valuable in investigating the dynamics of key protein structures. The increased availability of three-dimensional structures has in fact aided this process, as more detailed questions about the interrelationships between, for example, active sites and interacting protein side chains, can be addressed. A goal yet to be realized for biochemists is to bridge the dynamic changes in structure and the static endpoints provided by crystallography or thermodynamic analysis.

For PTPases, the conformational change due to ligand binding is restricted to the movement of a flexible loop that can be described as a hinged loop movement (Stuckey et al., 1994; Jia et al., 1995). This loop, termed the WpD loop or movable loop, harbors the essential general acid (an aspartic residue, Figures 5 and 6). It has been found that after substrate/oxyanion binding, the loop folds over the active site to position the Asp residue close to the scissile oxygen of the substrate for efficient proton transfer. The crystal structures suggest that the movable loop in the *Yersinia* PTPase has two distinct conformations (Figure 7). In the ligand-free enzyme form, the WpD loop exists in an "open" conformation and there is negligible interaction between the WpD loop and the PTP loop. In the ligand-bound enzyme form, the WpD loop adopts a "closed" state and Asp356 on the WpD loop makes hydrogen bond with the ligand. Because crystallization may preferentially stabilize one conformation over the other, it is important to determine the conformational state in solution for the *Yersinia* PTPase. Furthermore, because crystallographic studies have revealed the extent of the loop movement in the *Yersinia* PTPase, it is also important to determine the dynamics of this flexible loop motions.

Time-resolved fluorescence spectroscopy and anisotropy decay studies are well-established methods to characterize the protein conformational state. Furthermore, fluorescence lifetimes and rotational correlation times of intrinsic probes, such as tryptophan or tyrosine residues, belong to the

same time scale of local protein motions and fluctuations (Beechem and Brand, 1985; Karplus and Petsko, 1990). Thus, a description of processes involving the dynamics of the microenvironment of the fluorophore as well as those concerning the protein as a whole is accessible using dynamic fluorescence methods. The *Yersinia* PTPase possesses a desirable property for spectroscopic investigation because it contains only one Trp residue in a polypeptide of 51 kDa (Zhang et al., 1992). More importantly, the singular tryptophan residue Trp354 is invariant among all PTPases and is located at one of the hinge positions of the same flexible loop (WpD loop) as is the general acid Asp356 that undergoes a major conformational change when tungstate or sulfate is bound to the enzyme. Steady state spectroscopic and biochemical studies have shown that Trp354 acts as an intrinsic active site probe whose spectral properties are sensitive to structural perturbations introduced by mutations or ligand binding (Zhang and Wu, 1997). Thus, the *Yersinia* PTPase provides a unique and ideal system to study the conformational properties of the movable loop.

The conformational state of the movable loop in the *Yersinia* PTPase in aqueous solution was examined by steady-state ultraviolet resonance Raman (UVRR) spectroscopy (Juszczak et al., 1997). The UVRR data indicate that in solution the ligand-free *Yersinia* PTPase exists as an equilibrium mixture of almost equal populations of two tryptophan rotamer structures with $\chi^{2,1}$ dihedral angles of –4° and –90°. The two rotamers have been attributed to the "closed" and "open" WpD loop conformations, respectively. Time-resolved fluorescence anisotropy experiments were performed in order to determine the dynamics of the loop movement. The fluorescence anisotropy decay data indicate that, in the absence of ligand, the *Yersinia* PTPase alternates between an open WpD loop and a closed WpD loop form with a rate constant of 2.6×10^8 s^{-1} (Juszczak et al., 1997). The fact that the WpD loop exists in approximately equal populations of closed and open conformations and that these two conformers interchanges with rate constant close to that of the free diffusion of such a loop segment suggest that there is little energy required for the loop movement. Such a property is exactly what one would expect for an efficient enzyme that allows facile substrate binding and product release. These results differ from the classic "induced-fit" view in which the WpD loop remains open until closure is triggered by ligand binding. In the ligand-bound state, the Raman data indicate that the equilibrium population of the WpD loop is dominated by the closed conformation. The fluorescence anisotropy decay data suggest that the rate of loop opening is reduced significantly and that there is little motion on the nanosecond time scale. Thus, oxyanion binding stabilizes the closed conformation.

Substitution of the active site nucleophile Cys403 by a Ser residue abolishes the *Yersinia* PTPase activity (Guan and Dixon, 1990). Crystallographic studies reveal no noticeable differences between structures of the wild-type *Yersinia* PTPase and the C403S mutant. Surprisingly, the Raman and fluorescence anisotropy decay experiments indicate that the ligand-free and ligated C403S mutant *Yersinia* PTPase remain predominantly in the WpD loop closed conformation in solution and that there is no loop motion during the time course of the dynamic measurements. The apparent pK_a of the side chain of Cys403 is 4.7 (Zhang and Dixon, 1993), so that it exists as the thiolate anion at physiological pHs. In the wild-type enzyme, a delicate balance between the electrostatics of the PTP loop and the WpD loop may have been evolved. The binding pocket must facilitate the binding of anionic

phosphotyrosine without stabilizing the closed loop conformation in the ligand-free state where Asp356 of the WpD loop may become a pseudo-ligand. The net effect of the C403S mutation is to replace the negatively charged thiolate with an uncharged polar OH group. This may result in either a loss of repulsive potential between the Cys403 thiolate and the Asp356 carboxylate and/or the formation of a hydrogen bond or water-bridged hydrogen bond between Ser403 and Asp356, leading to the collapse of the loop and stabilization of the closed conformation. These results are consistent with the observations that in the C403S mutant the Trp354 fluorescence increases 340% over the wild-type *Yersinia* PTPase, and that its accessibility by fluorescence quenchers is significantly reduced (Zhang and Wu, 1997). These data also highlight the extreme susceptibility of the PTPase active site conformation and dynamics even to a conservative mutation such as Cys to Ser.

Is the flexibility of the WpD loop movement intrinsically related to the catalytic activity of the *Yersinia* PTPase? In the case of triosephosphate isomerase, the flexible loop motion was found to be independent of the ligation state as determined by solid state NMR techniques, and the rate of the loop motion was determined similar to that of the catalytic turnover number (Williams and McDermott, 1995). Interestingly, it appears that the WpD loop dynamics in the *Yersinia* PTPase is ligand dependent. In the absence of ligand, the wild-type *Yersinia* PTPase alternates between the closed and open forms with a rate constant of 2.6×10^8 s^{-1}, which is more than five orders of magnitude faster than the k_{cat} value. After ligand binding the rate of loop opening is dramatically reduced, resulting in a mostly closed conformation on a time scale of less than 10 ns. It is important to determine the loop dynamics in the presence of ligand, as this may be directly linked to catalysis. In addition, the catalytic activity among PTPases differ by more than six orders of magnitude (Zhang et al., 1992), and it has been postulated that the wide range of catalytic rates may be due to the dynamics of the loop motion and that these dynamics may be partially determined by the residues of the β-turn within the WpD loop (Schubert et al., 1995). In fact, the amino acid sequence of the movable loop is quite divergent except for the WpD sequence that includes the catalytic aspartic acid and a tryptophan near the hinge position of the loop (Figure 6). To investigate the relationship between loop dynamics and catalysis in the *Yersinia* PTPase, it may be quite insightful to systematically perturb the structure of the loop region and study how it affects the loop dynamics and PTPase catalysis. Because the fluorescence anisotropy decay technique is limited to the time scale of nanoseconds, NMR techniques may be more suitable, which can measure dynamic processes ranged from millisecond to picosecond time scale (Palmer, 1993; Zhao et al., 1996).

D. The Rate-Limiting Step

Because the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases effect catalysis through a covalent thiophosphate enzyme intermediate, the catalyzed reaction must be composed of at least two chemical steps, that is, formation and breakdown of the phosphoenzyme intermediate. The phosphoryl group in the substrate is first transferred to the nucleophilic active site thiolate group of the enzyme to form the E^*-P (k_3), which is then hydrolyzed by water (k_4) (Scheme 1). As intrinsic heavy atom kinetic isotope effects are observed for the phosphatase-catalyzed *p*NPP hydrolysis, substrate binding and the

putative loop conformational change do not contribute significantly to the rate-determining step. If the net rate of intermediate breakdown is slower than that of intermediate formation, one would expect a "burst" of *p*-nitrophenol production using *p*NPP as a substrate. On the other hand, if the rate of E^*-P formation is the rate-limiting step, no burst should be observed. It appears that the rate-limiting step for the low-molecular-weight, phosphatase-catalyzed aryl phosphate hydrolysis reactions is E^*-P hydrolysis (Zhang and Van Etten, 1991a; Zhang et al., 1995a). Supporting evidence include that up to 74% of the enzyme can be trapped as a covalent adduct using ^{32}P-labeled *p*NPP (Zhang, 1990) and that such an intermediate can be prepared in sufficient amount for ^{31}P NMR analysis (Wo et al., 1992). The pre-steady state burst kinetics experiments have allowed the determination of individual rate constants directly associated with the formation (k_3) and breakdown (k_4) of the phosphoenzyme intermediate, and evaluation of the burst kinetics of the wild-type and mutant phosphatases has led to the clarification of specific contributions of active site residues to the individual steps in the phosphatase-catalyzed reaction (Wu and Zhang, 1996; Zhao and Zhang, 1996).

There have been some disagreement in the literature regarding the nature of the rate-limiting step in reactions catalyzed by protein tyrosine phosphatases and dual specificity phosphatases. Burst kinetics has been observed with the *Yersinia* PTPase (Zhang et al., 1995b) and rat PTP1 (Zhang, 1995) at 3.5°C. It was concluded that under these conditions the rate-limiting step corresponds to E^*-P hydrolysis. The observed rate constant for E^*-P formation in the *Yersinia* PTPase- and PTP1-catalyzed reaction is around 200 to 350 s^{-1} at 3.5°C, which is barely within the detection limit of a typical stopped-flow spectrophotometer. Thus, one would not expect to detect the burst phase at 30°C. Indeed, no burst phase was observed during the pre-steady state of PTP1-catalyzed hydrolysis of *p*NPP at 30°C. The lack of a burst phase in the pre-steady state was attributed to an indication of rate-limiting E^*-P formation (Lohse et al., 1997). In another study, the receptor-like PTPase LAR-catalyzed hydrolysis of a phosphotyrosine-containing peptide substrate was examined by rapid chemical quench flow technique at room temperature (Cho et al., 1992). The rate of E^*-P formation was found 15 times faster than its decay. The pre-steady state kinetics was also examined in the SHP–1 (a SH2 domain containing PTPase) catalyzed *p*NPP hydrolysis (Wang and Walsh, 1997). Both SHP–1 and SHP–2 possess low basal phosphatase activity due to intramolecular inhibition by the SH2 domains and the C-terminal sequence segment downstream of the catalytic domain (Pei et al., 1994; Pluskey et al., 1995). Engagement of the SH2 domains with specific phosphotyrosine-containing peptides results in an enzyme conformation that is more active (Lechleider et al., 1993; Dechert et al., 1994; Pluskey et al., 1995). Furthermore, elimination of both SH2 domains or the C-terminal segment also enhances the activity of the catalytic domain (Zhao et al., 1993; Pei et al., 1994). Interestingly, the full-length SHP–1 that exists in an auto-inhibited form did not show a burst, while the 50-fold more activated catalytic domain of SHP–1 did exhibit burst kinetics. However, in this case the rate-limiting step has been attributed to the release of inorganic phosphate from the $E^*{\cdot}P_i$ complex. Like its counterparts, *Yersinia* PTPase and PTP1 in the PTPase subfamily, VHR has been studied extensively as the prototype for the dual specificity phosphatases. When pre-steady-state kinetic measurements of the VHR-catalyzed hydrolysis of *p*NPP were conducted at pH 7 and 30°C, no significant burst phase was observed (Denu et al., 1995). The inability to detect significant

burst phase in the VHR reaction led to the conclusion that enzyme phosphorylation being the rate-limiting step. In apparent contrast, burst kinetics was observed for the VHR-catalyzed *p*NPP hydrolysis in a separate study at pH 6 and 30°C, and the rate of E^{*}-P formation was found to be 4.5-fold faster than its hydrolysis (Zhang et al., 1995c).

It is difficult to reconcile these apparent discrepancies regarding the nature of the rate-limiting step in the PTPase and dual specificity phosphatases reaction. However, it is clear that in cases where burst kinetics has been observed, the rate of E^{*}-P formation is only a few folds faster than its hydrolysis (Zhang et al., 1995b,c; Wang and Walsh, 1997). Thus, it is perhaps not too surprising to see the disparity in these observations. In contrast, in the case of the low-molecular-weight phosphatases, the rate of E^{*}-P formation is 30- to 40-fold faster than its hydrolysis (Zhang and Van Etten, 1991a; Zhang et al., 1995a; Wu and Zhang, 1996). Differences in experimental conditions such as pH, temperature, and buffer components may differentially influence the individual rate constant of the two chemical steps that may result in a change in the rate-limiting step. Furthermore, the identity of the rate-limiting step can also be dependent on the choice of substrate used. For example, the dual specificity phosphatase cdc25B-catalyzed hydrolysis of 3-*O*-methylfluorescein phosphate exhibited burst kinetic pattern, while no burst was detected when *p*NPP was used as a substrate (Gottlin et al., 1996). Finally, the nature of the rate-limiting step can also be affected by the form of enzyme that is used in the experiment (Wang and Walsh, 1997). In future experiments all possibilities need to be considered and further studies are required to sort out these differences. It is possible that the rate-limiting step in reactions catalyzed by the PTPases and the dual specificity phosphatases may vary and dependent on the specific enzyme, substrate, and experimental conditions.

VI. CONCLUSIONS AND PERSPECTIVES

The level of tyrosine phosphorylation, and thus the strength and duration of the signal transmitted, are balanced by the opposing action of PTKs and PTPases. This balance is perturbed in transformed cells and metabolic disorders. Although the precise details of PTPases signaling remain to be established, the evidence presented in this review indicates that PTPases are involved in a number of oncogenic and disease processes. Unfortunately, a detailed understanding of the role played by PTPases in cellular signaling processes has been hampered by the absence of PTPase-specific agents. Further understanding of the specific functional roles of PTPases in cellular signaling requires definition of physiological substrates for each individual member of the PTPase family and detailed understanding of structural features that control PTPase substrate specificity. Kinetic analyses of PTPase catalytic domains with phosphopeptides as substrates indicate that PTPases display only modest amino acid sequence specificity at the peptide level (Zhang et al., 1993a,b; Cho et al., 1993; Hippen et al., 1993; Ruzzene et al., 1993; Zhang et al., 1994d; Dechert et al., 1995). However, recent genetic and biochemical evidence suggest that *in vivo* PTPases can exhibit extremely stringent substrate specificity (Herbst et al., 1996; Garton et al., 1996; Liu et al., 1996; Black and Bliska, 1997; Flint et al., 1997). Understanding the molecular basis for protein tyrosine dephosphorylation by PTPases will open doors to new experimental approaches (such as the

creation of PTPases with altered catalytic and regulatory properties and the design and development of specific PTPase inhibitors) that will elucidate mechanisms by which these enzymes control cell functions. As our understanding of the substrate specificity and the factors that control this specificity increases, efforts in the area of rational design of inhibitors that are targeted to specific PTPases should become possible. Inhibitors that specifically target particular PTPases may not only serve as useful intracellular probes of PTPase action but may ultimately provide a molecular basis for the design of medicinally useful agents.

Mechanistic and structural investigations from a number of laboratories have generated a great deal of information about how the PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases catalyze phosphomonoester hydrolysis. Although there are only limited sequence similarities among the three subfamilies of phosphatases, it is apparent that many of the essential structural features are conserved among these diverse phosphatase families and that a common chemical mechanism for substrate hydrolysis is utilized by these diverse catalysts. The detailed mechanism employed by these phosphatases to effect phosphate monoester hydrolysis is beginning to emerge. Measurements of heavy atom kinetic isotope effects have led to the initial characterization of the transition states for members of PTPases, the dual specificity phosphatases, and the low-molecular-weight phosphatases. Interestingly, measurable differences can be detected in the structures of the transition states of these different phosphatase-catalyzed reaction even though they utilize the same chemical mechanism. Further studies with specific mutant phosphatases may reveal the structural basis for these differences in the transition state. It is also important to address the mechanism by which the active site Arg residues stabilize the dissociative transition state. PTPases catalysis involves a conformational change that is restricted to the movement of a flexible loop that can be described as a hinged loop movement. It remains to be established whether similar loop movement also occurs in the dual specificity phosphatases and the low-molecular-weight phosphatases. Furthermore, it also remains to be established whether the loop dynamics is intrinsically linked to substrate binding and catalysis. The availability of three-dimensional structures of several phosphatase catalytic domains will facilitate detailed studies of structure/function relationships. Because members of each phosphatase subfamily share a catalytic domain with considerable amino acid sequence identity and structure similarity, in-depth kinetic and structural analysis of a representative number of phosphatases from each group of the PTPase superfamily will most likely continue to yield insightful mechanistic information that may be applicable to the rest of the family members.

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