Dual-specific protein phosphatases in the *Archaea*

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Abstract

Three distinct families of PTPs, the conventional (cPTPs), low molecular weight (LMW PTPs), and Cdc25 PTPs, have converged upon a common catalytic mechanism and active site sequence, mainly, the phosphate-binding loop encompassing the PTP signature motif (H/V)**C**$(X)_5$**R**(S/T) and an essential Asp residue on a surface loop. There is little sequence similarity among the three families of phosphatases. All known LMW PTP remove phosphoryl groups esterified to the hydroxyl amino acid: tyrosine, whereas all members of the Cdc25 family are dual-specificity protein phosphatases that dephosphorylate all the hydroxyl amino acids: tyrosine, serine and threonine. The cPTP family primarily functions as tyrosine phosphatases, but it also includes dual-specific members.

ORFs encoding potential cPTPs have been identified in five archaeal species: *Methanobacterium thermoautotrophicum, Methanococcus jannaschii*, *Thermococcus kodakaraensis*, *Pyrococcus horikoshii*, and *S. solfataricus*. Only one has been partially characterized, *Tk*-PTP from *T. kodakaraensis*. Hence, our current body of knowledge concerning the functional properties and physiological roles of these enzymes remains fragmented.

The genome of *S. solfataricus* encodes a single conventional protein tyrosine phosphatase, SsoPTP. SsoPTP is the smallest known archaeal PTP (18.3 kDa) with a primary amino acid sequence that conforms to the cPTP protein tyrosine phosphatase paradigm, $HCX_5R(S/T)$.

Relatively little is known about its mode of action – whether it follows the conventional PTP mechanism or employs a different route for catalysis – or its physiological role.

ORF *sso2453* from the genome of *Sulfolobus solfataricus,* encoding a protein tyrosine phosphatase, was cloned and its recombinant protein product, SsoPTP, was expressed in *E. coli* and purified by immobilized metal affinity chromatography. SsoPTP displayed the ability to dephosphorylate protein-bound phosphotyrosine as well as protein-bound phosphoserine/phosphothreonine. SsoPTP hydrolyzed both isomers of naphthyl phosphate, an indication of dual specificity. The four conserved residues within the presumed active site sequence: Asp^{69}, His^{95}, Cys^{96}, and Arg^{102}, and the invariant Gln^{139} residue were essential for catalysis, as it was predicted for the established members of the PTP family in both bacteria and eukaryotes. A substrate trapping protein variant, SsoPTP-C96S/D69A, was constructed to isolate possible SsoPTP substrates present in *S. solfataricus* cell lysates. Several potential substrates were isolated and identified by mass spectroscopy.

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CHAPTER I

INTRODUCTION

Protein phosphorylation-dephosphorylation

Protein phosphorylation-dephosphorylation represents nature's most dynamic mechanism for regulating the functional properties of proteins (Kennelly 2001). Unlike allosteric regulation, which requires the addition of a binding domain for the allosteric ligand as well as some means of communication between the two domains of a protein, protein phosphorylation is relatively simple (Wurgler-Murphy *et al.* 2004). The introduction of a phosphorylation site (amino acid residue containing a hydroxyl side chain) at a strategic location on a protein can transform an 'ordinary protein' into one whose functional properties respond in a dynamic fashion to a covalent modification event (Kennelly 2003). Protein phosphorylation, which is catalyzed by protein kinases, can cause physical transformation of the protein resulting in significant alteration of functional properties such as catalytic efficiency and affinity for substrates (Kennelly 1999) (Figure 1-1).

Protein phosphorylation acts as a reversible on/off switch – once a protein is phosphorylated, it can be restored to its initial, dephosphorylated state through the hydrolytic activity of protein phosphatases. This ability to restore a protein to its original state enables its reuse when once again needed by the cell (Kennelly 2002). The action of multiple protein kinases and protein phosphatases on a given phosphorylation site can generate correspondingly complex output patterns. This interplay among protein kinases and protein phosphatases enables

sets of phosphoproteins to be linked together to form sophisticated information processing networks capable of coordinating a range of molecular processes (Wurgler-Murphy *et al.* 2004).

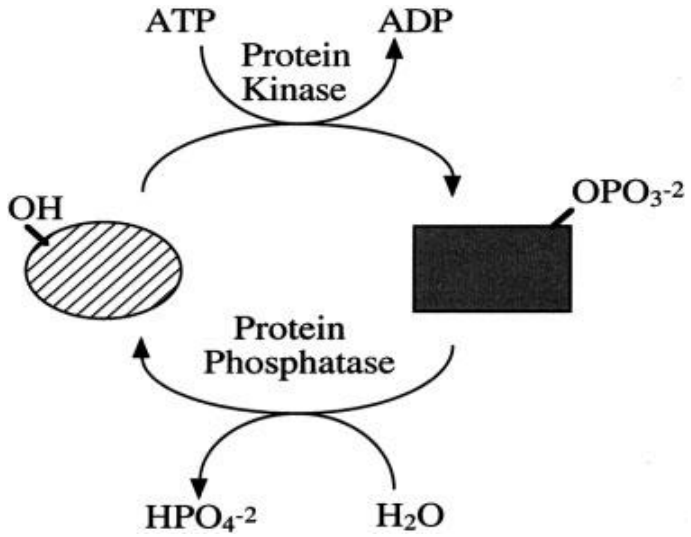

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Figure 1-1. Illustration of protein phosphorylation-dephosphorylation mechanism. Protein kinases transfer a phosphate group from a high energy phosphodonor, such as ATP, to a hydroxyl side chain of serine, theonine, and tyrosine residues on a protein substrate (represented in oval and rectangular shapes). Once a protein is phosphorylated, it undergoes physical transformation that results in altering its functional properties such as catalytic efficiency, affinity to substrates, or subcellular localization. The two different states of the protein substrate are represented in hatched oval and shaded rectangular. Protein phosphatases catalyze the removal of the phosphate group, added by protein kinases, and thus, restore protein substrates to their original unmodified state.

History

Protein phosphorylation-dephosphorylation was first discovered in higher eukaryotes during studies on the hormonal regulation of glycogen metabolism by Fischer and Krebs in the 1950s (Fischer *et al.* 1989). The first eukaryotic regulatory protein kinase to be discovered was phosphorylase kinase, followed by the identification and characterization of the cAMP-dependent protein kinase (Walsh *et al.* 1968; Krebs 1989). Consequently, protein phosphorylation-dephosphorylation mechanism was widely considered to be a late-emerging mechanism for regulating molecular events to meet the needs of higher eukaryotes with multiple differentiating cells (Kennelly 2003; Wurgler-Murphy *et al.* 2004). However, in the 1980s, it was discovered that the isocitrate dehydrogenase in the bacterium *E. coli* was regulated by phosphorylation (Garnak *et al.* 1979; LaPorte 1993). The discovery of the first bacterial protein kinase, the isocitrate dehydrogenase kinase/phosphatase, and the histidine kinases of the two component system established protein phosphorylation-dephosphorylation as a regulatory mechanism in prokaryotes as well (Cortay *et al.* 1988; Klumpp *et al.* 1988; Stock *et al.* 1989).

The bacterial isocitrate dehydrogenase kinase/phosphatase and the two component system displayed no resemblance in either structure or catalytic function to the well-established eukaryotic kinase and phosphatase families, which led to the belief that the origins of the eukaryotic and prokaryotic phosphorylation mechanism were distinct and exclusive to each domain (Hunter 1987; Kennelly *et al.* 1996; Kennelly 2001). This view was challenged by the discovery of a protein phosphatase in bacteriophage λ that shared 15-20% amino acid sequence identity with the eukaryotic protein serine/threonine phosphatase family (the PPP family) and, subsequently, the eukaryotic-like protein tyrosine phosphatase, YopH, from the bacterium *Yersinia pestis* (Cohen *et al.* 1989; Guan *et al.* 1990).

More discoveries soon followed in the early 1990s that included bacterial and archaeal protein kinases and protein phosphatases that shared structural and functional similarities to the members of eukaryotic protein kinase and protein phosphatase families. Examples of these early discoveries included eukaryotic protein kinases in the bacterial species: *Myxococcus xanthus* (Munoz-Dorado *et al.* 1991), *Anabaena* PCC 7120 (Zhang 1993), *Streptomyces coelicolor* A3(2), and *Streptomyces lividans* (Matsumoto *et al.* 1994; Urabe *et al.* 1995); PPP family of protein phosphatases from the archaeon *Sulfolobus solfataricus* (Kennelly *et al.* 1993), and the bacterium *E. coli* (Missiakas *et al.* 1997); PPM family of protein phosphatases from the bacterium *Bacillus subtilis* (Duncan *et al.* 1995); a conventional PTP with dual specificity (DSP) from the cyanobacterium *Nostoc commune* sp. strain UTEX 584 (Potts *et al.* 1993); and a low molecular weight (LMW) PTP from the bacterium *S. coelicolor* A3(2) (Umeyama *et al.* 1996).

Continued discoveries of additional protein kinases and protein phosphatases have been made possible by the availability of complete genome sequences for many prokaryotes (1085 complete genomes: 78 archaeal and 1007 bacterial; and 2564 genomes in progress: 49 archaeal and 2515 bacterial) (Entrez 2010). By using genome homology searches, many open reading frames (ORFs) of genes encoding a potential kinase or protein phosphatase have been identified (Kennelly 2003). About 2-4% of genes in a typical eukaryotic genome are accounted for by those encoding protein kinases and protein phosphatases (Manning *et al.* 2002).

Protein phosphatases families

Numerous studies have reinforced the concept that protein phosphorylation is a common means for the regulation of cellular processes, is ancient in origin, and is probably a universal covalent modification among organisms (Cohen 2002; Hunter 2007; Moorhead *et al.* 2009). The side chains of nine amino acids have the potential to form covalent bonds with a phosphoryl group (tyrosine, serine, threonine, cysteine, arginine, lysine, aspartate, glutamate and histidine) with serine, threonine and tyrosine phosphorylation being predominant in eukaryotic cells, where they play key regulatory roles (Moorhead *et al.* 2009). Proteomic analyses of three bacterial species identified over sixty proteins that were phosphorylated on serine, threonine and tyrosine of approximately 69, 22 and 9% distribution, respectively (Macek *et al.* 2007; Macek *et al.* 2008; Soufi *et al.* 2008). In humans, proteins with phosphorylation on serine, threonine and tyrosine residues are approx. 86.4, 11.8 and 1.8%, respectively (Olsen *et al.* 2006).

Five protein phosphatase families have been identified and characterized at the structural and functional level (Table 1-1). Two families are specialized in the hydrolysis of phosphoester bonds on phosphoserine and phosphothreonine residues *via* a common, single step mechanism (direct hydrolysis using an activated water molecule): the PPP and the PPM families. The low molecular weight protein tyrosine phosphatase family, LMW PTPs, and a subset of the conventional protein tyrosine phosphatase family, cPTP, are comprised of specialized phosphatases that dephosphorylate phosphotyrosine residues. The Cdc25 family and a subset of the cPTP family consist of phosphatases that are capable of hydrolyzing phosphoserine, phosphothreonine, and phosphotyrosine. For this reason they are oftentimes called 'dual specific' phosphatases or DSPs. The cPTPs, LMW PTPs, and Cdc25 families share, a common

active site motif, Cys-Xaa$_5$-Arg, and two-step ping-pong mechanism involving the formation of a phosphocysteinyl enzyme intermediate (Kennelly 2001; Kennelly 2003).

Family	Prominent members	Phylogenetic distribution	Catalytic mechanism	Active site signature motif
PPP	PP1, PP2A, PP2B	Archaea Bacteria Eucarya	direct hydrolysis	GDXHG, GDXXDRG, GNH(E/D)
PPM	PP2C, SpoIIE	Archaea Bacteria Eucarya	direct hydrolysis	(S/T)DGXX(D/E/N), D(D/N)X(T/S)
cPTP	PTP1B, YopH	Archaea Bacteria Eucarya	ping-pong, Cys-P enzyme intermediate	D-$X_{\approx 30}$-HCX_5R(S/T)
LMW PTP		Archaea Bacteria Eucarya	ping-pong, Cys-P enzyme intermediate	CX_5R-$X_{85\text{-}105}$-DP
Cdc25		Eucarya	ping-pong, Cys-P enzyme intermediate	D-$X_{\approx 45}$-CX_5R

Table 1-1. Distribution of Protein phosphatase families. Examples of representative members of each family, their phylogenetic distribution, choice of catalytic mechanism, and their conserved signature motifs are listed under each column, respectively. Adapted from Kennelly et al. (2001).

Protein serine/threonine phosphatases

The PPP family

The members of the PPP family represent the most abundant form of serine/threonine phosphatases in eukaryotes (Shi *et al.* 1998). The major representatives of the eukaryotic PPP family include protein phosphatases 1 (PP1), PP2A, PP2B (also called calcineurin) (Mumby *et al.* 1993; Barton *et al.* 1994; Shenolikar 1994). Recent additions to the PPP family include: PP4, PP5, PP6 and PP7 (Cohen *et al.* 2005; Stefansson *et al.* 2006). Members of the PPP family have been found in bacteria such as *E. coli* (Missiakas *et al.* 1997) and *Microcystis aeruginosa* (Shi *et al.* 1999)), archaeons such as *Sulfolobus solfataricus* (Kennelly 2003), and viruses such as bacteriophage λ (Cohen *et al.* 1989).

Members of the PPP family share a common catalytic domain of approximately 300 amino acids in length characterized by a set of three conserved sequence motifs containing essential catalytic residues: GDXHG (motif I), GDXXDRG (motif II), and GNH(E/D) (motif III) (Barton *et al.* 1994; Lohse *et al.* 1995; Shi *et al.* 1998). The catalytic domain of the PPP family is highly conserved in eukaryotes (≥34% sequence identity), which ranks the PPPs among the most highly conserved of all eukaryotic enzymes (Cohen *et al.* 1990; Kennelly 2001). These catalytic subunits are also highly conserved among representatives from viral, bacterial, and archaeal organisms, which suggest that members of this family employ a general mechanism for catalysis that is shared by phosphohydrolases of many kinds (Kennelly 1999). Two of the more recently identified members of the PPP family, PP4 and PP6, have also remarkable conservation of regulatory subunits across species (Moorhead *et al.* 2009).

The eukaryotic members of the PP1/2A/2B family are metalloenzymes that bind a pair of divalent metal ions (King *et al.* 1984; Chu *et al.* 1996). The proposed chemical mechanism proceeds *via* a single step that involves activation of a water molecule by one or more of the metal ions for direct attack and hydrolysis of the phosphoprotein substrate (Lohse *et al.* 1995; Barford 1996). X-ray crystallography has revealed that many of the amino acid residues within the three conserved sequence motifs participate in metal ion binding (Egloff *et al.* 1995; Goldberg *et al.* 1995). Some members of the PPP family, including calcineurin (Pallen *et al.* 1983; Chan *et al.* 1986) and recombinant forms of PP1 (MacKintosh *et al.* 1996), exhibited dual specificity *in vitro;* however, there is no direct evidence of physiological relevance of this activity *in vivo* (Kennelly 2001). For many years the lack of apparent substrate specificity of PPP protein phosphatases puzzled scientists. Later, it was revealed that substrate selectivity of eukaryotic PPP members is modulated through the addition of distinct targeting and regulatory domains, rather than through discrimination at the active site itself (Hubbard *et al.* 1993; Pawson *et al.* 1997).

Several bacterial and viral members of the PPP family have been identified and characterized. Examples include: PrpA and PrpB from *E. coli* (Missiakas *et al.* 1997), SppA from *Streptomyces coelicolor* A3(2) (Umeyama *et al.* 2000), PP1-cyano1 and PP1-cyano2 from *Microcystis aeruginosa* PCC7820 and UTEX2063, respectively (Shi *et al.* 1999), PP-lambda from bacteriophage λ gt.10 (Cohen *et al.* 1988; Cohen *et al.* 1989). The degree of sequence similarity between the bacterial PPPs and the eukaryotic PPPs is much lower (19%) than that between archaeal PPPs and their eukaryotic counterparts.

Unlike the eukaryotic metalloenzymes, the bacteriophage lambda and bacterial PPPs require the addition of an exogenous metal ion such as Mn^{2+} for catalytic activity, and are

resistant to potent inhibitors of PP1 and PP2A such as okadaic acid or microcystin (Kennelly 2001). In addition, nearly all members of the bacterial PPPs as well as the bacteriophage lambda protein phosphatase display dual specific activity *in vitro* (Shi *et al.* 1998; Kennelly 2001).

ORFs encoding potential members of the PPP family were identified in several archaeal genomes (Table 1-2), and three have been characterized: PP1-arch1 from *Sulfolobus solfataricus* (Kennelly *et al.* 1993; Leng *et al.* 1995), PP1-arch2 from *Methanosarcina thermophila* TM-1 (Oxenrider *et al.* 1993; Solow *et al.* 1997), and Py-PP1 from *Pyrodictium abyssi* TAG11 (Mai *et al.* 1998). The level of sequence similarity of archaeal PPPs with eukaryotic PPPs is high, ranging from 27-31% over the catalytic core domain (Barton *et al.* 1994),

Both PP1-arch1 and PP1-arch2 protein phosphatases displayed no catalytic activity towards phosphotyrosine-containing substrates *in vitro*, indicating that these archaeal PPPs are serine/threonine specific protein phosphatases. Similar to the bacterial PPPs, archaeal PPPs require an exogenous metal ion such as Mn^{2+}, Ni^{2+}, or Co^{2+} to support activity, with Mn^{+2} being the preferred metal ion. Archaeal PPPs do not display the nanomolar sensitivity of the eukaryotic PP1 and PP2A to toxic secondary metabolites such as okadiac acid or tautomycin (Cohen *et al.* 1991). However, PP1-arch2 and Py-PP1 are mildly sensitive to microbial toxins, whereas PP1-arch1 is unaffected (Kennelly *et al.* 1993; Oxenrider *et al.* 1993; Solow *et al.* 1997; Mai *et al.* 1998). The current knowledge of the physiological roles of archaeal PPPs is limited, since almost all studies of these enzymes were performed on the recombinant products of their cloned genes (Kennelly 2001).

The PPP family

Organism	ORF/protein	Length		Motif		
				I	II	III
			Consensus …	o*oGDoHG$_{61}$*o	ooooGDooDRG$_{91}$	oorGNHE$_{121}$
A. pern.	*APE0777*	266 aa		MGDIHG$_{6}$DY	MFLLGDYIDRG$_{38}$	MLRGNHE$_{69}$
A. fulg.	*AF1822*	268 aa		tvVGDVHa$_{35}$DI	aIFLGDYaDRG$_{61}$	LLRGNHE$_{88}$
M. thphl.	PP1-arch2	268 aa		VmIGDIHG$_{49}$NL	FLFLGDYVDRG$_{79}$	LLRGNHE$_{108}$
P. abyssi	Py-PP1	302 aa		LvaGDtHG$_{38}$yp	VVFLGDYVDRG$_{68}$	LLRGNHE$_{97}$
S. solf.	PP1-arch1	293 aa		VFVGDtHG$_{40}$AI	IVFLGDYVDRe$_{69}$	VLRGNHE$_{104}$
T. volc.	*TVN1195*	229 aa		IiYsDIHa$_{11}$NL	aIFLGDVVDyG$_{38}$	gVmGNHd$_{61}$

The PPM family

Organism	ORF/protein	Length		Motif	
				VIII	XI
			Consensus …	ooo+*DGoodoo*	Dnotoooxo
T. volc.	*TVN0703*	218 aa		FIICTDGVsDnLS	DdaTIIkVyV

The cPTP family

Organism	ORF/protein	Length		Motif
			Consensus …	DX$_{27}$ooHCxagxxRTg
M. *therm.*	mt1586	233 aa		DX$_{33}$agaCagGvmRSl
M. *jann.*	MJ0215	159 aa		DX$_{26}$VVsCigGhgRTG
M. *jann.*	MJ1098	254 aa		DX$_{34}$YFHCykykrRTs
M. *jann.*	MLECL20	147 aa		DX$_{26}$VVsCigGhgRTG
P. *hori.*	PH1732	146 aa		DX$_{26}$YIHCygGsgRSG
S. *solf.*	sso2453	161 aa		DX$_{24}$LVHCvgGigRTG
T. *kod.*	Tk-PTP	147 aa		DX$_{26}$LIHCmgGlgRSG

The LMW PTP

Organism	ORF	Length		Motif
			Consensus …	ooClGNiCRSX$_{99}$DP
A. fulg.	AF1361	135 aa		FVCIhNtaRSX$_{88}$KD
M. therm.	*mt1355*	130 aa		FICrnNsgRSX$_{83}$PD

Table 1-2. Salient features of select known or potential archaeal members of the PPP, PPM, cPTP, and LMW PTP families of protein phosphatases. Symbols used to denote the various features are as follows: uppercase letters, universally conserved amino acid residues; lowercase letters, highly conserved amino acid residues; ‘o’, positions conserving non-polar residues; ‘*’, positions conserving polar residues; ‘x’, any amino acid; and ‘+’, positions conserving small residues with near neutral polarity. Shown above each consensus sequence are the regions from the potential archaeal proteins postulated to correspond thereto. Residues that match the consensus are shown in uppercase letters. Abbreviations used for the organisms: *A. pern.*, *Aeropyrum pernix*; *A. fulg.*, *Archaeoglobus fulgidus*; *M. therm.*, *M. thermoautotrophicum*; *M. jann.*, *Methanococcus jannaschii*; *P. hori.*, *Pyrococcus horikoshii*; *S. solf.*, *Sulfolobus solfataricus*; *T. volc.*, *Thermoplasma volcanium*; aa, amino acids. Adapted from Kennelly (2003).

The PPM family

The most prominent representatives of the PPM family of serine/threonine phosphatases are protein phosphatase 2C (PP2C) and pyruvate dehydrogenase phosphatase in eukaryotes (Barford 1996; Bork *et al.* 1996). The eukaryotic PP2C was first identified as a Mn^{2+}-dependent enzyme whose sequence has no resemblance to other protein phosphatases (Tamura *et al.* 1989). All members of the PPM family require an exogenous divalent metal ion, such as Mg^{2+}, for their catalytic activity, and are resistant to microcystin, okadaic acid and other classic inhibitors of the PPP family (Das *et al.* 1996; Moorhead *et al.* 2009).

The active site domain of PPMs spans a region approximately 290 amino acid residues in length marked by 11 conserved motifs containing 9 essential residues, four of which are aspartates that co-ordinate metal ions necessary for catalysis (Bork *et al.* 1996; Das *et al.* 1996; Moorhead *et al.* 2009). Although members of the PPM family do not share significant sequence similarity with the PPPs (Tamura *et al.* 1989; Lawson *et al.* 1993), X-ray crystallography revealed a striking similarity between their active site geometries, indicative of convergence upon a common catalytic mechanism (Das *et al.* 1996).

Several eukaryotic members of the PPM family have been identified and characterized in organisms ranging from mammals (Tamura *et al.* 1989; Wenk *et al.* 1992; Lawson *et al.* 1993) to plants (Stone *et al.* 1994) to insects (Dick *et al.* 1997). All eukaryotic PP2Cs appear to be monomeric proteins, while pyruvate dehydrogenase phosphatase is a heterodimer (Lawson *et al.* 1993). Unlike the PPPs, no targeting or regulatory domains for the PP2C subfamily of PPMs have been identified. However, studies showed that the sequences of some members of the PPM family include membrane-targeting motifs (Klumpp *et al.* 1994; Stone *et al.* 1994) and protein

kinase interaction sequences (Welihinda *et al.* 1998) in their N-terminal and C-terminal extensions. Eukaryotic PPMs are known to be involved in responding to environmental stresses such as anoxia, heat or osmotic shock (Maeda *et al.* 1993; Shiozaki *et al.* 1994; Gaits *et al.* 1997), and progression through the cell cycle (Tong *et al.* 1998; Cheng *et al.* 1999).

The bacterial members of the PPM family are considered the most prevalent form of serine/threonine phosphatases in the *Bacteria*. Approximately half of all bacterial genomes encode a known or potential PPM (Kennelly 2001; Kennelly 2002). A bacterial form of PP2C, SpoIIE, was identified in *Bacillus subtilis*, where it plays a role in sporulation (Duncan *et al.* 1995). Several other bacterial PPMs were also identified and characterized. Examples include: RsbP (Vijay *et al.* 2000), RsbU (Yang *et al.* 1996), RsbX (Yang *et al.* 1996), and PrpC (Obuchowski *et al.* 2000) from *Bacillus subtilis*; Slr1860/IcfG (Shi *et al.* 1999) from the cyanobacterium *Synechocystis* PCC6803; Stp1 (Mukhopadhyay *et al.* 1999) from *Pseudomonas aeruginosa*. Both SpoIIE and Slr1860/IcfG contain additional domains that contribute to their large size (Beuf *et al.* 1994; Kennelly 2001). While most bacterial species contain one or a few PPMs, two *Streptomyces* species have as many as 49 PPMs (Moorhead *et al.* 2009).

Experimental studies showed that some bacterial PPMs are highly specific for their protein substrates *in vitro*. For example, SpoIIE displayed no phosphatase activity when tested with phosphothreonine-altered SpoIIAA, its native phosphoserine-containing substrate, indicating remarkably strict selectivity (Duncan *et al.* 1995). Stp1 from *P. aeruginosa*, on the other hand, exhibited less selectivity as proved by its ability to dephosphorylate phosphocasein *in vitro* (Mukhopadhyay *et al.* 1999). Several bacterial PPMs exhibit dual specific phosphatase activity: PphA (Ruppert *et al.* 2002) and SynPPM3 (Li *et al.* 2005) from *Synechocystis* sp. stain PCC6803, and PrpZ (Lai *et al.* 2005) from *Salmonella enterica* serovar Typhi. Bacterial PPMs

have been shown to be involved in stress responses in *B. Subtilis* (Adler *et al.* 1997) and *M. xanthus (Treuner-Lange et al. 2001),* and the co-ordination of carbon metabolism in *Synechocystis* PCC6803 (Irmler *et al.* 2001).

A single ORF encoding an archaeal member of the PPM family of protein serine/threonine phosphtases, TvnPPM, has been identified in the genome of *Thermoplasma volcanium* (Table 1-2) (Kawashima *et al.* 2000). Thus far, it is the sole PPM member in the *Archaea.* A recombinant form of TvnPPM displayed dual specific phosphatase activity *in vitro,* ranking it the fourth member in the PPM family with dual specific capability (chapter VIII of this dissertation). TvnPPM showed a divalent metal ion-dependant phosphatase activity *in vitro*. The physiological role of TvnPPM in *T. volcanium* is yet to be determined.

Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) constitute a large and structurally diverse family of signaling enzymes that, together with protein tyrosine kinases, modulate the cellular level of tyrosine phosphorylation (Neel *et al.* 1997). In higher eukaryotes, an appropriate level of tyrosine phosphorylation is essential for regulating cell growth, differentiation, metabolism, progression through the cell cycle, cell-cell communication, cell migration, gene transcription, ion channel activity, the immune response, and apoptosis/survival decisions (Zhang 2003).

Three distinct families of PTPs have converged upon a common catalytic mechanism and active site sequence but share little sequence similarity: the conventional (cPTPs), low molecular weight (LMW PTPs), and Cdc25 PTPs (Kennelly 2002). The PTP signature motif (H/V)**C**(X)5**R**(S/T) is located in the catalytic domain sequence (Zhang 1998). Catalysis proceeds *via* a two-step mechanism (Guan *et al.* 1991) that employs the active site cysteine residue as a

nucleophile (Figure 1-2) (Zhou *et al.* 1994). In the first step, the thiol group of the active site cysteine carries out a nucleophilic attack on the substrate-bound phosphoryl group. This results in the transfer of the phosphoryl group from the substrate to the enzyme, forming a phosphocysteinyl enzyme intermediate. An invariant aspartic acid residue protonates the dephosphorylated substrate that then dissociates from the enzyme. In the second step, an activated water molecule hydrolyzes the cysteinyl phosphate group to generate inorganic phosphate and free enzyme (Kennelly 1999; Kennelly 2001). Invariant Arg, Asp and Gln residues play important roles in catalysis: Arg is involved in substrate recognition and stabilization of the transition state; Asp acts as a general base to protonate the leaving group and as a general acid to activate the water molecule participating in the second step (Zhang 1998; Kennelly 2001; Zhang 2003); Gln positions the water molecule for the direct attack on the phosphocysteine in the active site (Zhao *et al.* 1998).

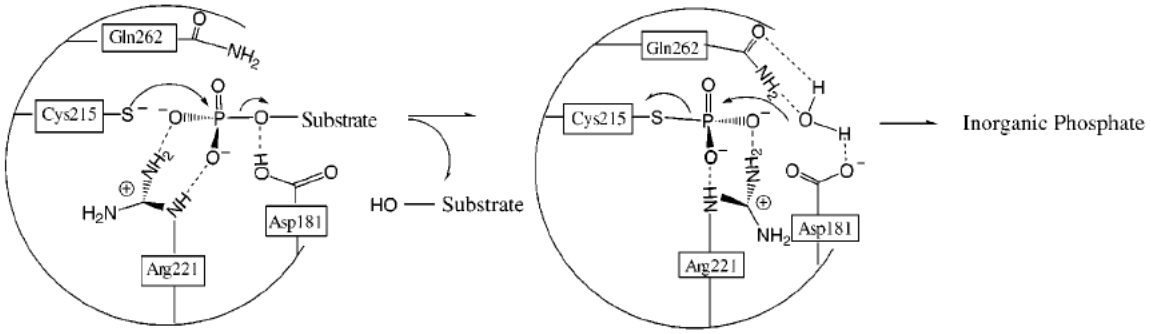

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Figure 1-2. The proposed chemical mechanism for the PTP family. Residues are numbered with reference to PTP1B (Xie *et al.* 2002).

The conventional PTP family

The members of the cPTP family constitute the most abundant form of protein phosphatases in the *Eukarya* (Charbonneau *et al.* 1992). The human genome encodes more than 100 cPTPs, in addition to 11 PTPs that are catalytically inactive and 16 that dephosphorylate non-protein substrates (1, 2 and 13 PTPs that phosphorylate glycogen, mRNA and phosphoinositides respectively) (Alonso *et al.* 2004).

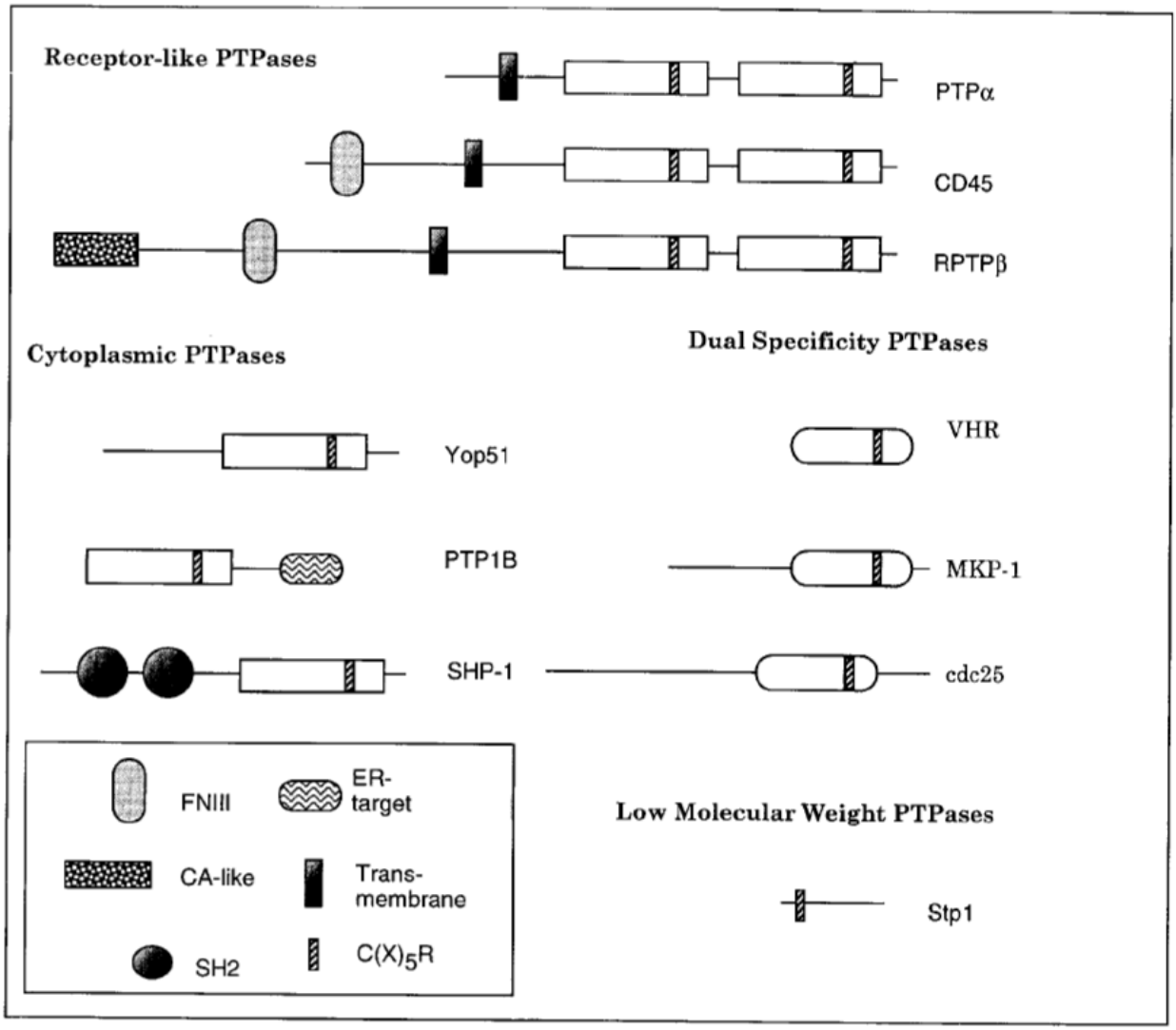
Members of the cPTP family contain an essential aspartic acid residue that is located 25-45 residues to the N-terminal side of the active site signature motif, which is located near the center of the catalytic domain, consisting of approximately 230 amino acid residues (Fauman *et al.* 1996; Tonks *et al.* 1996; Denu *et al.* 1998).

Members of the cPTP family can be classified into receptor-like and intracellular cPTPs (Figure 1-3) (Zhang 1998). The receptor-like cPTPs, such as CD45 (Charbonneau *et al.* 1988), PTPα (Zheng *et al.* 1992), and RPTPβ (Peles *et al.* 1995), contain an extracellular domain, a single transmembrane region, and one or more cytoplasmic domains, sometimes including tandem catalytic domains. The intracellular cPTPs, represented by PTP1B (Chernoff *et al.* 1990) from human placenta and YopH (Guan *et al.* 1990) from the bacterium *Yersinia pestis*, contain a single catalytic domain and various amino or carboxyl terminal extensions, including the macromolecule docking motifs such as SH2 domains, as in SHP-1 (Shultz *et al.* 1993), that may have targeting or regulatory functions (Figure 1-3) (Zhang 1998).

Members of the cPTP family are mainly tyrosine-specific phosphatases; however, several cPTPs possess the ability to dephosphorylate phosphoserine and phosphothreonine residues in addition to phosphotyrosine, and hence they are called DSPs (dual-specific phosphatases).

Examples include: the human VHR (Yuvaniyama *et al.* 1996), MKP-1(Chu *et al.* 1996), and KAP (Hannon *et al.* 1994) (Figure 1-3). The DSPs, which include all cPTPs that target nonprotein substrates, can be further divided into the MAPKP (mitogen-activated protein kinase phosphatase); slingshot; PRL (phosphatase of regenerating liver); atypical DSP; CDC14 (cell division cycle 14); the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Myers *et al.* 1997; Maehama *et al.* 1998); CEL-1, PIR1, and BVP RNA 5′-di- and tri-phosphatases (Takagi *et al.* 1997; Deshpande *et al.* 1999); and MTMs (myotubularins) (Moorhead *et al.* 2009).

One primary source of the difference in substrate specificity between strict PTPs and DSPs would appear to be the relative depth of their active site pockets (Barford *et al.* 1998; Denu *et al.* 1998). The deep pockets of strict PTPs (~9Å) render them incapable of dephosphorylating phosphoserine and phosphotheonine, thus restricting their hydrolytic capability to the long side chain of tyrosine (Kennelly 2001).



[fair use]

Figure 1-3. Classification of protein tyrosine phosphatases. Abbreviations used are as follows: FNIII, fibronectin type III, ER, endoplasmic reticulum, CA, carbonic anhydrase, SH2, src-homology 2 (Zhang *et al.* 1998)

Several bacterial members of the cPTP family have been identified and characterized: YopH (Guan *et al.* 1990) from the pathogenic strains of *Yersinia*, SptP (Kaniga *et al.* 1996) from *Salmonella typhimurium*, MPtpB (Koul *et al.* 2000) from *Mycobacterium tuberculosis*, and IphP (Potts *et al.* 1993) from *Nostoc commune* UTEX584. The tyrosine specific phosphatase YopH plays an essential role in the virulence of *Yersinia*, which was responsible for the pandemic plague in medieval Europe popularly known as Black Death (Guan *et al.* 1990). Both SptP and MPtpB display tyrosine-specific activity and are essential for the virulence of their respective pathogens, whereas IphP is a dual specific phosphatase *in vitro* that has a role in the metabolism or sensory functions in the cyanobacterium *N. commune* (Shi *et al.* 1998).

Members of the cPTP family were also identified in several viruses: VH1 (Guan *et al.* 1991) from *Vaccinia*, I1L from *Myxoma*, and M1L from *Shope fibroma* (Mossman *et al.* 1995). These viral cPTP appear to be dual specific phosphatases *in vitro*.

ORFs encoding potential cPTPs have been identified in five archaeal species: *Methanobacterium thermoautotrophicum, Methanococcus jannaschii*, *Thermococcus kodakaraensis*, *Pyrococcus horikoshii*, and *S. solfataricus* (Table 1-2). Only two, *Tk*-PTP from *T. kodakaraensis*, and SsoPTP from *S. solfataricus*, have been characterized to any significant extent (Jeon *et al.* 2002; Kennelly 2003; Chu *et al.* 2007). SsoPTP, the sole cPTP encoded by the genome of *S. solfataricus*, was observed to exhibit dual specific phophatase activity *in vitro* (Dahche and Kennelly, this study), while *Tk*-PTP hydrolyzed free phosphotyrosine and phosphoserine, but not phosphothreonine (Jeon *et al.* 2002). *Tk*-PTP has yet to be tested for activity against protein-bound phosphoesters.

The crystal structure of SsoPTP has been determined and, to date, it is the only known structure for an archaeal PTP (Chu *et al.* 2007). SsoPTP shares low sequence similarity with other members of the cPTP family, with similarities mainly found in the secondary structure elements and active site sequence (Figure 1-4 and 1-5), with significant differences in the loop regions. However, comparison of the three-dimensional structure of SsoPTP with other known structures of cPTPs found in the Protein Data Bank (Berman *et al.* 2000) using Dali server (Holm *et al.* 1996) revealed several structures homologous with that of SsoPTP: Cdc14B (Gray *et al.* 2003), KAPt (Song *et al.* 2001), and VHR (Yuvaniyama *et al.* 1996). Each of these proteins exhibits dual-specific protein phosphatase activity and shares 21%, 27%, and 23% sequence identity with SsoPTP, respectively.

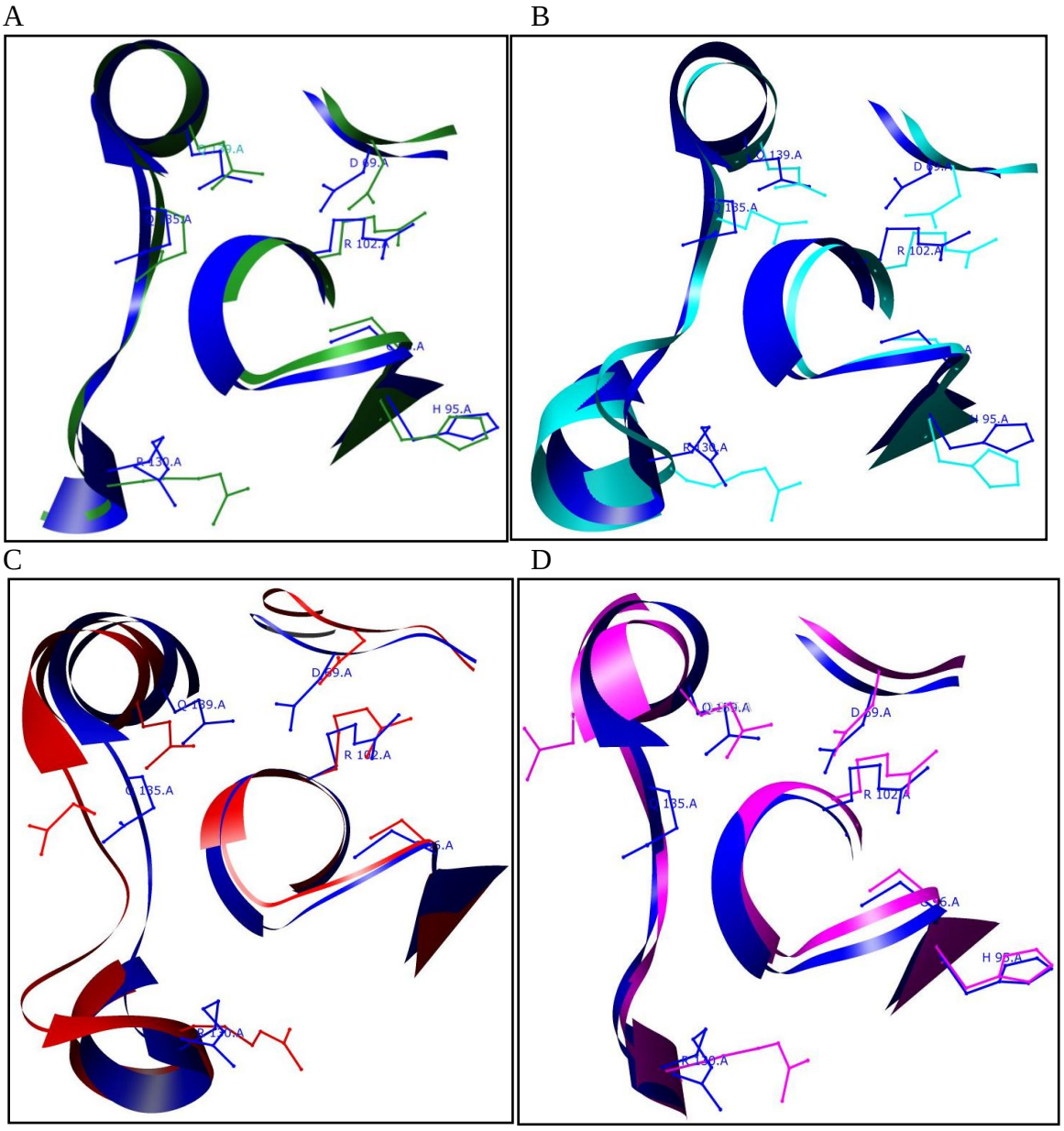
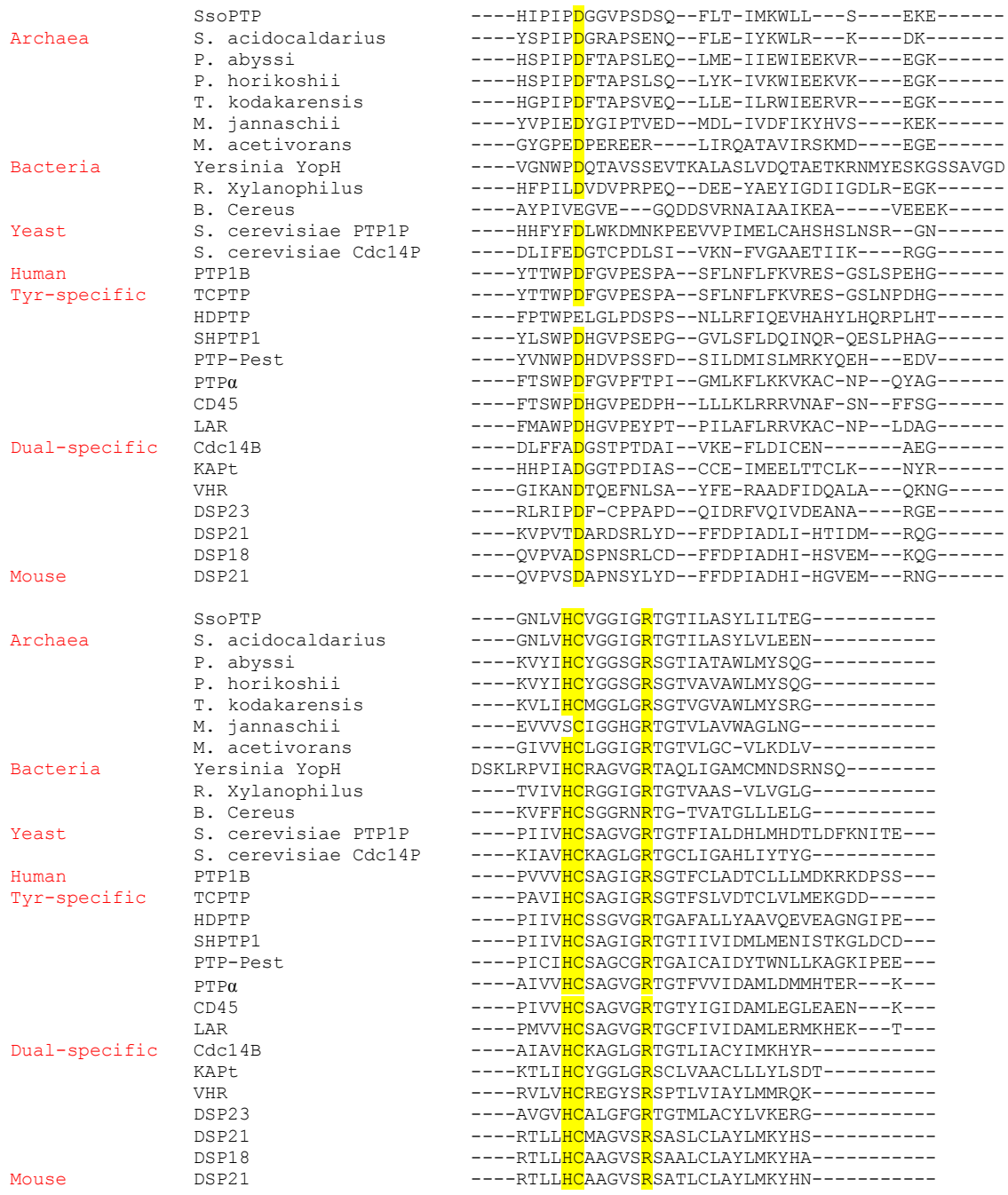


Figure 1-4. Active site three-dimensional structure alignment of SsoPTP, represented in blue ribbon with: (A) PTP1B, (B) Yersinia cPTP, (C) KAPt, and (D) Cdc14B. Catalytic residues are shown as ball and stick representation with numbering corresponding to SsoPTP: R^{130}, R^{102}, Q^{135}, Q^{139}, C^{96}, H^{95}, and D^{69}. Images were generated using CHIMERA.


SsoPTP ----HIPIPDGGVPSDSQ--FLT-IMKWLL---S----EKE------
Archaea S. acidocaldarius ----YSPIPDGRAPSENQ--FLE-IYKWLR---K----DK-------
P. abyssi ----HSPIPDFTAPSLEQ--LME-IIEWIEEKVR----EGK------
P. horikoshii ----HSPIPDFTAPSLSQ--LYK-IVKWIEEKVK----EGK------
T. kodakarensis ----HGPIPDFTAPSVEQ--LLE-ILRWIEERVR----EGK------
M. jannaschii ----YVPIEDYGIPTVED--MDL-IVDFIKYHVS----KEK------
M. acetivorans ----GYGPEDPEREER----LIRQATAVIRSKMD----EGE------
Bacteria Yersinia YopH ----VGNWPDQTAVSSEVTKALASLVDQTAETKRNMYESKGSSAVGD
R. Xylanophilus ----HFPILDVDVPRPEQ--DEE-YAEYIGDIIGDLR-EGK------
B. Cereus ----AYPIVEGVE---GQDDSVRNAIAAIKEA-----VEEEK-----
Yeast S. cerevisiae PTP1P ----HHFYFDLWKDMNKPEEVVPIMELCAHSHSLNSR--GN------
S. cerevisiae Cdc14P ----DLIFEDGTCPDLSI--VKN-FVGAAETIIK----RGG------
Human PTP1B ----YTTWPDFGVPESPA--SFLNFLFKVRES-GSLSPEHG------
Tyr-specific TCPTP ----YTTWPDFGVPESPA--SFLNFLFKVRES-GSLNPDHG------
HDPTP ----FPTWPELGLPDSPS--NLLRFIQEVHAHYLHQRPLHT------
SHPTP1 ----YLSWPDHGVPSEPG--GVLSFLDQINQR-QESLPHAG------
PTP-Pest ----YVNWPDHDVPSSFD--SILDMISLMRKYQEH---EDV------
PTPα ----FTSWPDFGVPFTPI--GMLKFLKKVKAC-NP--QYAG------
CD45 ----FTSWPDHGVPEDPH--LLLKLRRRVNAF-SN--FFSG------
LAR ----FMAWPDHGVPEYPT--PILAFLRRVKAC-NP--LDAG------
Dual-specific Cdc14B ----DLFFADGSTPTDAI--VKE-FLDICEN-------AEG------
KAPt ----HHPIADGGTPDIAS--CCE-IMEELTTCLK----NYR------
VHR ----GIKANDTQEFNLSA--YFE-RAADFIDQALA---QKNG-----
DSP23 ----RLRIPDF-CPPAPD--QIDRFVQIVDEANA----RGE------
DSP21 ----KVPVTDARDSRLYD--FFDPIADLI-HTIDM---RQG------
DSP18 ----QVPVADSPNSRLCD--FFDPIADHI-HSVEM---KQG------
Mouse DSP21 ----QVPVSDAPNSYLYD--FFDPIADHI-HGVEM---RNG------
SsoPTP ----GNLVHCVGGIGRTGTILASYLILTEG-----------
Archaea S. acidocaldarius ----GNLVHCVGGIGRTGTILASYLVLEEN-----------
P. abyssi ----KVYIHCYGGSGRSGTIATAWLMYSQG-----------
P. horikoshii ----KVYIHCYGGSGRSGTVAVAWLMYSQG-----------
T. kodakarensis ----KVLIHCMGGLGRSGTVGVAWLMYSRG-----------
M. jannaschii ----EVVVSCIGGHGRTGTVLAVWAGLNG----------
M. acetivorans ----GIVVHCLGGIGRTGTVLGC-VLKDLV----------
Bacteria Yersinia YopH DSKLRPVIHCRAGVGRTAQLIGAMCMNDSRNSQ--------
R. Xylanophilus ----TVIVHCRGGIGRTGTVAAS-VLVGLG----------
B. Cereus ----KVFFHCSGGRNRTG-TVATGLLLELG----------
Yeast S. cerevisiae PTP1P ----PIIVHCSAGVGRTGTFIALDHLMHDTLDFKNITE---
S. cerevisiae Cdc14P ----KIAVHCKAGLGRTGCLIGAHLIYTYG----------
Human PTP1B ----PVVVHCSAGIGRSGTFCLADTCLLLMDKRKDPSS---
Tyr-specific TCPTP ----PAVIHCSAGIGRSGTFSLVDTCLVLMEKGDD------
HDPTP ----PIIVHCSSGVGRTGAFALLYAAVQEVEAGNGIPE---
SHPTP1 ----PIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCD---
PTP-Pest ----PICIHCSAGCGRTGAICAIDYTWNLLKAGKIPEE---
PTPα ----AIVVHCSAGVGRTGTFVVIDAMLDMMHTER---K---
CD45 ----PIVVHCSAGVGRTGTYIGIDAMLEGLEAEN---K---
LAR ----PMVVHCSAGVGRTGCFIVIDAMLERMKHEK---T---
Dual-specific Cdc14B ----AIAVHCKAGLGRTGTLIACYIMKHYR----------
KAPt ----KTLIHCYGGLGRSCLVAACLLLYLSDT----------
VHR ----RVLVHCREGYSRSPTLVIAYLMMRQK----------
DSP23 ----AVGVHCALGFGRTGTMLACYLVKERG----------
DSP21 ----RTLLHCMAGVSRSASLCLAYLMKYHS----------
DSP18 ----RTLLHCAAGVSRSAALCLAYLMKYHA----------
Mouse DSP21 ----RTLLHCAAGVSRSATLCLAYLMKYHN----------

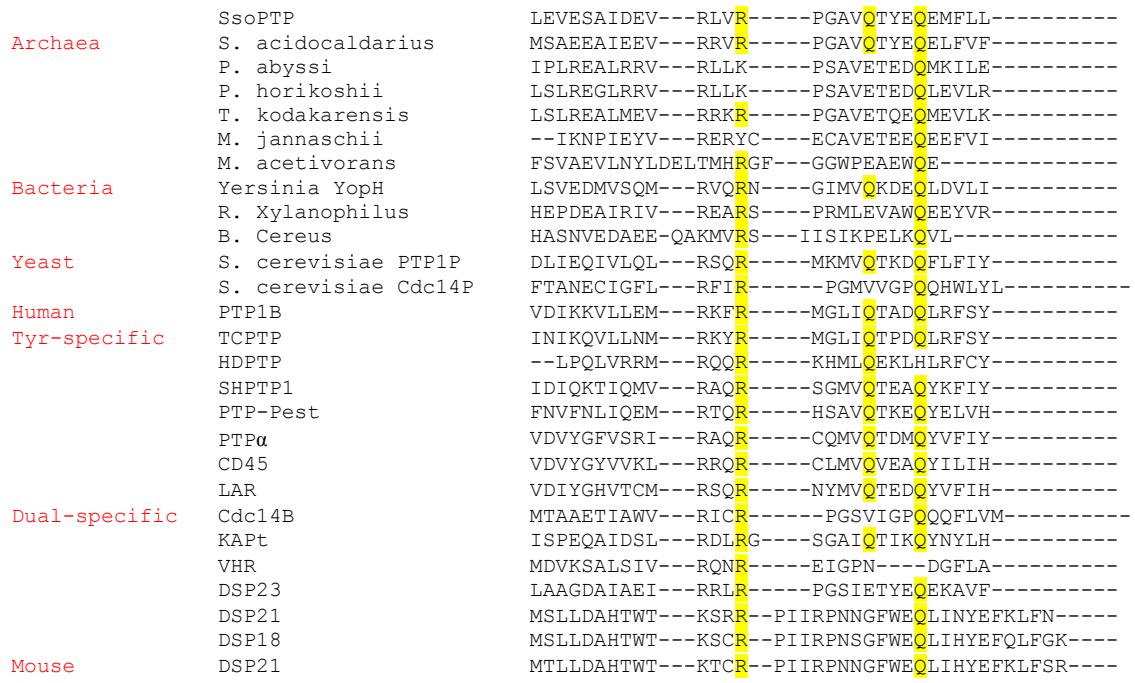


Figure 1-5. Primary amino acid sequence alignment of SsoPTP with various protein tyrosine phosphatses from the three domains, bacteria, archaea and eukarya. The conserved active site residues are highlighted in yellow.

The low molecular weight PTP family

The active site signature motif of the LMW PTP family is located near the extreme N-terminus of the catalytic domain (~150 residues), which is noticably smaller than that of the cPTPs (Shi *et al.* 1998). Unlike the cPTPs, the invariant aspartic acid residue is located to the C-terminal side of the catalytic cysteine, immediately N-terminal to a conserved proline residue (Fauman *et al.* 1996; Ramponi *et al.* 1997). Generally, the active site cysteine is preceded by an aliphatic hydrophobic residue such as valine or isoleucine rather than the histidine conserved amongst cPTPs (Kennelly 2001).

Members of the LMW PTPs are generally small (~18kDa), and share no significant sequence similarity with cPTPs. However, X-ray crystallography and NMR spectroscopy studies showed that both the active site configuration and secondary structures are remarkably similar (Logan *et al.* 1994; Su *et al.* 1994; Zhang *et al.* 1994). Thus, LMW PTPs and cPTPs appear to have converged upon a common catalytic mechanism and active site configuration (Ramponi *et al.* 1997). Alteration of the essential catalytic residues, Cys, Arg, and Asp, resulted in dramatic effect on catalytic activity as first observed for the cPTPs (Wo *et al.* 1992; Davis *et al.* 1994; Zhang *et al.* 1994; Denu *et al.* 1998). There has been no report of any dual specific activity in the LMW PTP family.

Members of the LMW PTPs have been characterized from mammals (Wo *et al.* 1992), plants (Bose *et al.* 1998), yeast (Mondesert *et al.* 1994; Ostanin *et al.* 1995), and bacteria (Li *et al.* 1996; Grangeasse *et al.* 1998; Vincent *et al.* 1999; Koul *et al.* 2000). In the *Archaea*, two ORFs for potential LMW PTPs have been identified in the genomes of *A. fulgidus*, and *M. thermoautotrophicum* (Table 1-2) (Kennelly 2003).

Several bacterial LMW PTPs share 30–40% sequence identity with their human counterparts, suggesting an ancient conserved function, but to date no clear role has been defined for LMW PTPs (Moorhead *et al.* 2009). In addition, several bacterial arsenate reductases have been discovered that contain the active site signature motif of LMW PTPs. These perform catalysis through the formation of an arsenocysteine intermediate analogous to the phosphocysteinyl intermediate of the LMW PTPs (Ji *et al.* 1992; Rosenstein *et al.* 1992; Liu *et al.* 1997). Hence, LMW PTPs are thought to have evolved from a bacterial arsenate reductase (Alonso *et al.* 2004; Tonks 2006; Moorhead *et al.* 2007).

The Cdc25 family

Members of the Cdc25 family constitute a specialized set of protein phosphatases with dual specific activity that targets adjacent phosphotyrosine and phosphothreonine residues on cyclin-dependant kinases in the *Eukarya* (Nilsson *et al.* 2000; Trinkle-Mulcahy *et al.* 2006). These enzymes are highly specific for their substrates, and their sole physiological function is to modulate progression through the eukaryotic cell cycle (Kennelly 2003).

Members of the Cdc25 family share similar active site signature motif and catalytic mechanism with other PTPs (Fauman *et al.* 1996; Gottlin *et al.* 1996; Denu *et al.* 1998). However, comparison of the three-dimensional structure of Cdc25 with members of the PTP family revealed that Cdc25 was topologically distinct, and closely resembled the sulfur metabolizing enzyme rhodanese, commonly found in bacteria (Fauman *et al.* 1998). No ORFs encoding potential Cdc25-type phosphatases thus far have been detected in either bacterial or archaeal genomes (Kennelly 2001; Kennelly 2003).

Protein phosphorylation in the *Archaea*

Recently, researchers have turned their focus to the members of the *Archaea* because of their extremophilic lifestyles and distinct phylogenetic status (Potters *et al.* 2003). Current evidence suggests that archaeal proteins undergo covalent modification by phosphorylation-dephosphorylation processes catalyzed by protein kinases and protein phosphatases. However, relatively little is known concerning the identities of the proteins targeted or the chemical nature, enzymatic mechanism, or physiological roles of their protein phosphoryl transfer enzymes (Kennelly 2003; Lower *et al.* 2003).

In 1980, the first evidence of phosphorylated polypeptides in the *Archaea* was reported in the extreme halophilic archaeon *Halobacterium salinarium* (Spudich *et al.* 1980). Lysates of *H. salinarium* cells that had been cultured in the presence of [^{32}P] phosphate were analyzed for phosphopolypeptides by SDS-PAGE/autoradiography. Two of the phosphoproteins detected were observed to be resistant to treatment with acid or hydroxylamine, indicative of a phosphoester bond (Spudich *et al.* 1980). A second report followed in 1984 in which Skorko established the existence of protein phosphorylation in the extreme acidothermophile *Sulfolobus acidocaldarius* (Skorko 1984). Several phosphorylated polypeptides were visualized on an SDS-PAGE gel that were labile to alkali and alkaline phosphatase, but not to snake-venom phosphodiesterase, consistent with linkage *via* a phosphomonoester bond. Phosphoamino acid analysis revealed that many of these phosphopeptides contained phosphoserine or phosphothreonine residues, suggesting the action of protein kinases. Using two-dimensional electrophoresis, Osorio and Jerez reported the presence of more than 20 ^{32}P-labelled proteins in *S. acidocaldarius* cells grown in the presence of [^{32}P] phosphate (Osorio *et al.* 1996).

In 1997, phosphotyrosine-directed antibodies were employed to detect phosphoproteins in several archaea, including the extreme acidothermophile *Sulfolobus solfataricus*, the extreme halophile *Haloferax volcanii*, and the anaerobic methanogen *Methanosarcina thermophila* TM-1 (Smith *et al.* 1997). More recently, three tyrosine-phosphorylated polypeptides were isolated and partially sequenced from lysates of the hyperthermophilic archaeon *Thermococcus kodakaraensis*, by affinity chromatography using a substrate-trapping mutant of a protein tyrosine phosphatase, *Tk*-PTP, (Jeon *et al.* 2002).

The abovementioned studies provide strong evidence that proteins within a broad spectrum of the *Archaea* are subject to covalent phosphorylation; however, little progress has been made in the elucidation of the identity of the archaeal proteins and/or the cellular processes that are targeted by this covalent modification. The first archaeal phosphoproteins of any type to be identified were homologs CheY and CheA in *H. salinarum* (Rudolph *et al.* 1995; Rudolph *et al.* 1995) and the methyltransferase activation protein (MAP), from the methanogenic archaeon *Methanosarcina barkeri* (Daas *et al.* 1996; Solow *et al.* 1998). Since then, only a few additional phosphoproteins have been identified (Eichler *et al.* 2005; Humbard *et al.* 2006).

The Phosphorylation Site Database was established to assemble and facilitate direct access to information from the primary scientific literature concerning members of the domains *Archaea* and *Bacteria* that have been reported to undergo covalent phosphorylation on serine, threonine, and/or tyrosine residues (Wurgler-Murphy *et al.* 2004). To date, the database contains 84 entries for protein phosphorylation in *Archaea*, including 76 from *Halobacterium salinarum* (Aivaliotis *et al.* 2009). Other entries include a putative phosphohexomutase (Sso0207) from *S. solfataricus* P2 (Ray *et al.* 2005); the Beta-1 subunit of the 20S proteasome, psmB1, from *H. volcanii* (Humbard *et al.* 2006); two cell division control protein 6, mthCdc6-1 and mthCdc6-2

from *Methanothermobacter thermoautotrophicus* (Grabowski et al. 2001); protein serine kinases Rio1 and Rio2 from *Archaeoglobus fulgidus* (LaRonde-LeBlanc *et al.* 2004); protein serine kinase SsoPK2 and protein serine/threonine kinase SsoPK3 from *S. solfataricus* P2 (Lower *et al.* 2003).

Sulfolobus solfataricus, a model archaeon

Extremophilic archaeons have adapted to thrive in habitats of extreme temperature, pH, salinity, and/or pressure, which has stimulated a lot of interest, aimed at understanding the adaptations to their lifestyles at the molecular level. The phylogenetic juxtaposition of the *Archaea* relative to the bacterial as well as eukaryotic domains has also motivated a lot of studies on evolutionary relatedness among the three domains (Ciaramella *et al.* 2002). The hyperthermoacidophilic archaeon *Sulfolobus solfataricus* represents one of the best-studied species of *Archaea*, and it has developed into an important model system for molecular, biochemical and genetic studies.

S. solfataricus, which belongs to the phylum Crenarchaeota, grows optimally at 80°C (60-92°C) and pH 2-4, and are found in sulfur-rich environments. *S. solfataricus* strain P2 (DSM 1617) was originally isolated from Pisciarelli, Italy (Zillig *et al.* 1980). *S. solfataricus* is a strict aerobe that can grow lithoautotrophically by oxidizing sulphur or ferrous ions (Brock *et al.* 1972) as well as chemoheterotrophically on a variety of organic compounds (sugars like glucose, galactose, arabinose, sucrose, etc., as well as amino acids or peptides) as carbon and energy sources. *S. solfataricus* can be easily cultured and maintained in the laboratory and cells can be frozen at -80°C to be preserved for posterity (Grogan 1989).

The complete genome of *S. solfataricus* P2 has been sequenced (She *et al.* 2001), which has contributed to the development of genetic and biochemical tools to examine this organism at the molecular level on a cellular scale. The genome size of *S. solfataricus* is about 2.99 Mb and encodes about 2900 proteins, 33% of which are unique to *Sulfolobus*, whereas 40% have homologues in other members of the *Archaea* (6.5% exclusively found in the *Archaea*), 12% have homologues in the *Bacteria* but not the *Eukarya*, and 2.3% in the *Eukarya* but not the *Bacteria*. A further 25% are shared with both domains (Ciaramella *et al.* 2002). *S. solfataricus* has been considered as the most 'eukaryotic-like' archaeon owing to its similarities with eukaryotes at the molecular level (Woese *et al.* 1985), which draws further attention to study this organism.

Over the few past years, *S. solfataricus* has become the center of intensive studies. Functional genomic approaches have been applied to study *S. solfataricus*, including transcriptomics, proteomics, and comparative genomics (Verhees *et al.* 2003; Snijders *et al.* 2006). Several *in vitro* assay systems have been established for *S. solfataricus* to analyze aspects of information processing in hyperthermophiles, such as replication, transcription, or translation, (Bell *et al.* 2001; Kelman *et al.* 2005; Barry *et al.* 2006; Ring *et al.* 2007). In addition, many of its proteins have been crystallized; including the conventional protein tyrosine phosphatase, SsoPTP (Chu *et al.* 2007). The development of genetic tools to enable the study of gene functions and the potential to perturb *S. solfataricus* system has also been developed; for example, a virus-based shuttle vector with a reporter gene has been developed to allow high-level gene expression of heterologous and homologous recombinant and tagged proteins (Jonuscheit *et al.* 2003; Worthington *et al.* 2003; Albers *et al.* 2006; Wagner *et al.* 2009). A study on the effects of oxidative stress (hydrogen peroxide treatment) revealed the upregulation

of genes and phosphorylation of many proteins (Maaty *et al.* 2009) in *S. solfataricus*. Recently, the *Sulfolobus* systems biology (SulfoSYS) project has been established to study the effect of temperature variation on the central carbohydrate metabolism of *S. solafataricus* (Zaparty *et al.* 2010).

Specific aims and significance of the project

ORFs encoding potential cPTPs have been identified in five archaeal species: *M. thermoautotrophicum, M. jannaschii, T. kodakaraensis, P. horikoshii*, and *S. solfataricus* . Only one has been partially characterized, *Tk*-PTP from *Thermococcus kodakaraensis* KOD1. Hence, our current body of knowledge concerning the functional properties and physiological roles of these enzymes remain fragmented.

The genome of *S. solfataricus* encodes a single conventional protein tyrosine phosphatase, SsoPTP. SsoPTP is the smallest known archaeal PTP (18.3 kDa) with a primary amino acid sequence that conforms to the cPTP protein tyrosine phosphatase paradigm, $HCX_5R(S/T)$. Relatively little is known about its mode of action – whether it follows the conventional PTP mechanism or employs a different route for catalysis – or its physiological role.

This project aims at characterizing SsoPTP in an attempt to pinpoint the similarities and differences in its specificity and chemical mechanism with those of established members of the cPTP family, as well as identifying potential substrates of SsoPTP endogenous to *S. solfataricus*.

The specific aims of the work outlined herein are:

1. **To determine the substrate specificity of SsoPTP *in vitro*:**

    a. Is SsoPTP specific for phosphotyrosine residues or does it also dephosphorylate phosphoserine and phosphothreonine residues?

    b. Is the active site wide enough to enable SsoPTP to hydrolyze α-naphthyl phosphate efficiently?

2. **To determine the chemical mechanism for SsoPTP:**

    Are the following residues essential for catalysis: Asp^{69}, His^{95}, Cys^{96}, Arg^{102}, Arg^{130}, Gln^{135}, and Gln^{139}?

3. **What are the possible physiological substrates of SsoPTP *in vivo*?**

CHAPTER II

MATERIALS AND METHODS

Materials

All custom oligonucleotides, Sephadex G-75 or G-25 beads for gel filtration, lysozyme from chicken egg white, Malachite green reagent, Angiotensin I, goat anti-mouse IgG (whole molecule)-alakaline phosphatase antigen specific antibody, Bromochloroindolyl phosphate (BCIP), and a 5% (w/v) solution of partially dephosphorylated casein were purchased from Sigma (St Louis, MO). *Pfu Turbo* DNA polymerase, *Pfu Ultra* DNA Polymerase, BL21-CodonPlus (DE3)-RIL cells, and a QuikChange II Site-Directed mutagenesis kit were purchased from Stratagene (LaJolla, CA). The QIAquick PCR purification and QIAprep spin miniprep kits were from Qiagen (Valencia, CA). Expression vectors pET-29b and pET-21d were from Novagen (San Diego, CA). *Escherichia coli* α-select cells, dNTPs mix, and His-Catch metal chelating cellulose (Ni^{+2} precharged) were from Bioline (Taunton, MA). Restriction enzymes and the catalytic subunit of the cAMP-dependent protein kinase (PKA) were from New England Biolabs (Beverly, MA). Genomic DNA from *Sulfolobus solfataricus* P2 was from the American Type Culture Collection (ATCC) (Manassas, VA). [^{32}P] ATP was from Perkin Elmer Life Sciences (Beverly, MA). *Escherichia coli* expressing the *lyn* protein-tyrosine kinase as a GST fusion protein was the gift of Professors Marietta Harrison and Harry Charbonneau of Purdue University (West Lafayette, IN). SDS-PAGE pre-stained standards and EZ load DNA molecular rulers were purchased from Bio-Rad (Hercules, CA). Coomassie protein assay reagent, *p*-nitrophenyl phosphate, and bovine serum albumin (BSA) were purchased from Pierce Biotechnology (Rockford, IL). Sequencing grade, modified trypsin, T4 DNA ligase, Kemptide,

and shrimp alkaline phosphatase (SAP) were purchased from Promega (Madison, WI). Myelin basic protein (MBP), and Immobolin-P PVDF membranes was from Millipore (Bedford, MA). Protease inhibitors cocktail was purchased from Research Products International Corp. (Mt. Prospect, IL). NHS-activated Sepharose 4 Fast Flow and Sephadex 200 10/300 GL column were purchased from Amersham (GE) Biosciences (Piscataway, NJ). Scintisafe 30% liquid scintillation fluid was purchased from Fisher Scientific (Pittsburgh, PA). Raytide was purchased from Oncogene Science (Cambridge, MA). P81 phosphocellulose filter paper and 3MM filter paper were from Whatman (Hillsboro. Oregon). Anti-phosphotyrosine antibody from mouse was purchased from Biosource (Camarillo, CA). All other reagents were from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Instrumentation

Polymerase chain reaction (PCR) was performed on a PCR Sprint Thermal Cycler with 24-well x 0.2 ml sub-ambient block from Thermo Fisher Scientific Inc. (Waltham, MA). A Fisher Scientific Marathon 12KBR centrifuge (Pittsburgh, PA) or a Sorvall RC-5B refrigerated superspeed centrifuge with SS-34 rotor from Du Pont Instruments (Memphis, TN) or an Eppendorf centrifuge Model 5415D with a 45° fixed-angle rotor at 16,100 rcf (13,200 rpm) (Westbury, NY) or an Optima L-90K ultracentrifuge with a 50 Ti fixed angle rotor from Beckman Coulter (Brea, CA) were all used to pellet cells or perform centrifugal separation on samples. A UV-visible recording spectrophotometer Model UV-260 from Shimadzu Scientific Instruments, Inc. (Columbia, MD) was used to measure the absorbance of liquid samples. An Accumet Basic AB15 pH meter from Fisher Scientific (Pittsburgh, PA) was used to measure the pH of solutions. To disrupt bacterial or *S. solfataricus* cells, a Sonifier cell disruptor Model

W185 from Heat Systems-Ultrasonics (Plainview, NY) was used. To grow *E. coli* or *S. solfataricus* cells, an Innova Model 4080 incubator shaker from New Brunswick Scientific Co. (Edison, NJ) or a Max Q mini 4450 shaker from Barnstead Lab-line (Melrose Park, IL) were used. An LS 6500 multi-purpose scintillation counter from Beckman Coulter (Atlanta, GA) was used to measure radioactivity in samples. To detect radioactivity, a survey meter Model 3 from Ludlum measurements (Sweetwater, TX) was used. Gels were scanned using a Bio-Rad Molecular Imager ChemiDoc XRS (170-8070) with Quantity One Software. Electronic autoradiography was performed on a Packard Instant Imager (Meriden, CT). A Waters 650 advanced protein purification system (FPLC) from Millipore (Milford, MA) was used for in-gel filtration chromatography.

Cloning of SsoPTP

Primer design

The forward and reverse primers were designed with 5'-tails containing recognition sites for two restriction enzymes, *Nco*I or *Sal*I, respectively. The restriction sites were designed to be in-frame with the pET-21d expression vector. The sequences of the forward and reverse primers are the following respectively, and the *Nco*I and *Sal*I recognition sequences are underlined:

5′-AGTCCATGGTAATGTATTGGGTTAGAAGGAAAACC-3′ and

5′-GTAATTGTCGACAGAATTCGAGTAGATGTTTTTCAAC-3′.

Polymerase Chain Reaction (PCR)

Open reading frame *sso2453* encoding SsoPTP was amplified via PCR in a 50 µl reaction containing 10 pmol forward and reverse primers, 350 ng of *S. solfataricus* P2 genomic DNA as a template, 10 nmol of each dNTPs, 1x *Pfu Ultra* reaction buffer, and 3.75 units of *Pfu Ultra* DNA polymerase. A standard three-step PCR cycle was used; an initial denaturation step of one cycle at 95°C for 10 minutes was set to denature the template DNA followed by thirty cycles of denaturation (94°C for 30 seconds), annealing (57°C for 30 seconds), and extension (72°C for 1 minute). The final step was set for 5 minutes at 72°C to finish extending all PCR products. The resulting PCR products were purified using QIAquick PCR purification kit following manufacturer's protocol. A 200 µl portion of buffer PBI (binding buffer containing guanidine hydrochloride and isopropanol) was mixed with an aliquot (40 µl) of the PCR samples, transferred to a QIAquick spin column and the mixture centrifuged for 30 to 60 seconds at 10, 000 x *g*. The flow-through was discarded and 750 µl of buffer PE was then added to the column and centrifugation performed as before. The flow-through was discarded. The column was centrifuged for an additional one minute and then transferred to a clean 1.5 ml Eppendorff tube. To elute adherent DNA, 50 µl water or buffer EB (10 mM Tris-Cl pH 8.5) was added to the center of the column, and the column was then centrifuged for 1 minute as before.

Restriction enzyme digestion

Following elution from the spin column, PCR products (~80 ng) were digested with 10 units each of the restriction enzymes *Nco*I and *Sal*I in a volume of 50 µl containing 5 µl reaction buffer #3 (50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol), and 5 µl of 10x BSA. The mixture was incubated overnight at 37°C. The digested PCR products were

then purified by the QIAquick PCR purification kit following manufacturer's protocol (see above).

Ligation

The digested PCR products were ligated into expression vector pET-21d. Before ligation, the expression vector pET-21d was digested with the same restriction enzymes used for digesting SsoPTP PCR products mentioned above, followed by treatment with shrimp alkaline phosphatase (SAP) to remove the phosphate group from the resulting 5′-termini. This was done to prevent ligation of the ends of linearized pET-21d vector backbone, which could result in unproductive recircularization and/or concatemer formation. The restriction-digested pET-21d (~6 µg) was incubated with 6 units of SAP at 37°C for 15 minutes in 1x SAP reaction buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 2 mM ATP, 10% PEG, 20 mM Dithiothreitol) in a final volume of 50 µl. SAP was then inactivated by heating at 65°C for 15 minutes. The reaction mixture was then centrifuged for 2 minutes at 12, 000 x g, and the supernatant was transferred to a clean microcentrifuge tube. The ligation reaction was then performed in a volume of 10 µl containing 1.5 units of T4 DNA ligase, 1 µl of 10x ligase buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100mM DTT and 10mM ATP), 100 ng (1 µl) of the SAP treated expression vector pET-21d, and 30 ng (8 µl) of the cleaned digested PCR products (DNA insert) or water as a control, incubated overnight at 4°C.

Transformation into *E.coli*

The ligation reaction mixture was used to transform chemically competent α-select *E. coli* cells. Chemically competent α-select *E. coli* cells were removed from -80°C freezer and

thawed on ice. An aliquot, 25 µl, of the cell suspension was transferred to a chilled 1.5 ml tube. A 4 µl portion of the ligation mixture was added to the cell suspension and the mixture gently swirled for a few seconds to mix, then incubated on ice for 30 minutes. After heating for 30 seconds at 42°C the tube was immediately chilled on ice for 2 minutes after which 300 µl of SOC medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 0.4% (w/v) glucose, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$) was added. The tube was incubated for 60 minutes at 37°C with shaking (~200 rpm). A 100 µl portion of transformation mixture were plated on LB agar plates containing ampicillin and incubated overnight at 37°C. Transformant colonies were picked and grown in 5 ml of LB (Luria-Bertani) broth containing 0.2 mg/ml of ampicillin and incubated overnight at 37°C with shaking (220-250 rpm).

Isolation of plasmid DNA (miniprep)

Plasmid DNA was isolated from 5 ml overnight cultures of *E. coli* using QIAprep spin miniprep kits following manufacturer's protocol. A 1 ml portion of each culture was transferred to a 1.5 ml Eppendorf tube. The tube was centrifuged for 5 minutes at 13, 000 x *g* to pellet bacterial cells. The pellet was resuspended in 250 µl Buffer P1 (resuspension buffer containing RNase A) and transferred to a microcentrifuge tube. Lysis buffer containing sodium hydroxide, 250 µl Buffer P2, was added and the tube sealed and then inverted 4-6 times. A 350 µl portion of Buffer N3 (neutralization buffer containing guanidine hydrochloride and acetic acid) was then added and the tube sealed and then inverted 4-6 times and followed by centrifugation at 13, 000 x *g* for 10 minutes in a microcentrifuge. The resulting supernatant was applied to a QIAprep spin column by pipetting. The column was centrifuged for 30-60 seconds at 13, 000 x *g* and the flow-through discarded. Next, 500 µl of Buffer PB (binding buffer) was applied and the column

centrifuged as before. This process was repeated using 750 µl Buffer PE (wash buffer). Each flow-though was discarded. Next, the column was centrifuged for an additional 1 minute to remove residual liquid, then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of water was added to the center of the column and the column was then centrifuged for 1 minute at 13, 000 x *g*. The eluant liquid, plasmid DNA, was collected and stored at -80°C. The orientation and sequence of the cloned gene was verified by sequencing the isolated plasmid at the Virginia Tech Bioinformatics Institute Core laboratory DNA sequencing facility (Blacksburg, VA). The plasmid construct (that is, SsoPTP cloned into pET-21d) was named sso2453.

Expression and purification of SsoPTP

Recombinant protein expression

Plasmid sso2453 was used to chemically transform *E. coli* BL21-CodonPlus (DE3)-RIL cells. Chemically competent *E. coli* BL21-CodonPlus (DE3)-RIL cells were thawed on ice and an aliquot, 50 µl, of the competent cell mixture was transferred to a pre-chilled 1.5 ml microcentrifuge tube. A 1 µl portion of plasmid DNA was added and the cell suspension gently mixed. The mixture was incubated on ice for 30 minutes then heated at 42°C for 30 seconds. Afterwards, the tube was immediately placed on ice. After 2 minutes on ice, 250 µl of SOC medium was added. The resulting cell transformation mixture was incubated at 37°C for an hour with shaking at 225-250 rpm. An aliquot, 150 µl, of the transformation mixture was then used to inoculate 5 ml of LB broth containing 0.2 mg/ml ampicillin and 34 µg/ml chloramphenicol. The culture was incubated overnight at 37°C with shaking at 225-250 rpm. The entire cell culture was

used to innoculate 250 ml of LB medium containing 0.1 mg/ml ampicillin, 34 µg/ml chloramphenicol, and 4 mM arginine. The culture was incubated at 37°C for 3 hours with shaking at 225-250 rpm. Expression of recombinant proteins was then induced by adding 200 µl of 1M IPTG (isopropyl-*β*-D-thiogalactopyranoside), and the culture incubated for another 3 hours at 37°C. The cells were then harvested by centrifugation for 15 minutes at 3000 x *g* and the resulting pellet was stored at -80°C until needed.

Purification of recombinant fusion proteins

SsoPTP protein phosphatase was produced as a recombinant fusion protein with a C-terminal hexahistidine tag. This tag enabled purification of SsoPTP by Immobilized Metal ion Affinity Chromatography (IMAC) (here, columns with immobilized Ni^{+2} ions were used). The following protocol was used to obtain purified SsoPTP. The bacterial cell pellet from an IPTG-induced culture was resuspended in 5 ml of starting buffer (50 mM MOPS pH 7, 150 mM NaCl, 20 mM imidazole) and then lysed by sonic disruption for 1 minute on ice. The resulting mixture was centrifuged for 20 minutes at 3000 x *g*. The supernatant liquid was removed and heated for 10 minutes at 55°C to denature endogenous *E. coli* proteins. The heated mixture was clarified by centrifugation for 10 minutes at 10, 000 x *g*. The resulting supernatant was added to a slurry, 1 ml bed volume, of Ni^{+2}-precharged metal chelating cellulose that had been previously equilibrated with 2 ml of starting buffer (50 mM MOPS pH 7, 150 mM NaCl, 20 mM imidazole). The mixture was rotated end-over-end at room temperature for 30 minutes, then centrifuged for 3 minutes at 500 x g. The supernatant liquid was removed and 10 bed volumes of starting buffer were then added. The mixture was agitated by rotating for 5 minutes, and centrifuged for 3 minutes at 500 x g and the supernatant liquid discarded. The washing process

was repeated twice. The beads were placed in a filter column and washed with 15 bed volumes of the starting buffer. To elute adherent proteins, 3 ml of elution buffer (50 mM MOPS pH 7, 150 mM NaCl, 500 mM imidazole) was applied and the eluate liquid was collected in 1 ml fractions. The eluate fractions were dialyzed against 20 mM Tris-HCl pH 7 overnight at 4°C. Protein concentration was determined by Bradford assay (Bradford, 1976). The purity of SsoPTP protein was ascertained by the presence of a single band on an SDS-PAGE gel.

Characterization of SsoPTP

Assays for protein phosphatase activity

p-nitrophenyl phosphate (*p*NPP)

Protein phosphatase activity was frequently assayed using *p*NPP as a substrate. Briefly, 0.5 µg of SsoPTP was mixed with 10 mM *p*NPP in a total reaction volume of 250 µl containing 100 mM MES pH 6. All assays were initiated by the addition of *p*NPP. The mixture was incubated for 10 minutes at 55°C. To stop the reaction, 750 µl of 0.5 M NaOH was added. If hydrolysis occurred, the color of the reaction mixture turns yellow because of the release of *p*-nitrophenolate (the result of dephosphorylating *p*NPP). The absorbance was measured at 410 nm using a spectrophotometer against a control reaction where SsoPTP was replaced with water. This assay was used to assess the optimal activity range for SsoPTP.

To determine optimal concentration of SsoPTP (that at which concentration remains in the linear range of the assay and about 10% of the substrate is consumed), 0.1, 0.3, 0.5, 0.8, 1, 1.5, 2, or 3 µg of SsoPTP was used.

To determine the optimal pH of the reaction, several different buffers were tested; each at a final concentration of 100 mM: sodium acetate pH 4.5 or 5.0; citric acid pH 4.5 or 5.0; MES pH 5.0 or 5.2 or 5.4 or 5.6 or 5.8 or 6.0 or 6.2 or 6.4 or 6.6 or 6.8 or 7.0; HEPES pH 7.0 or 7.4 or 8.2; Tris-HCl pH 7.4 or 8.2 or 8.8.

To determine the optimal time of assay, the reaction mixture was incubated for 5, 10, 15, 20, 25, 30, 35, or 40 minutes at 55°C. The effect of temperature on activity was determined by incubating the reaction for 10 minutes at the following temperatures: 25, 37, 42, 55, 65, 70, 75, 80, or 100°C.

The effect of ionic strength on the activity of SsoPTP was assessed by the addition of 50, 100, 250, 500, or 1000 mM NaCl to a reaction mixture containing 100 mM MES pH 6 and 0.5 µg SsoPTP.

All of the aforementioned assays were incubated for 10 minutes at 55°C, except the assay measuring optimal temperature of enzyme where reactions were incubated for 10 minutes at the specified temperatures.

To measure stability of SsoPTP, reactions were preincubated without *p*NPP at 25, 37, 42, 55, 65, 70, 75, or 80°C for 30 minutes. The tubes were then set on ice for 5 minutes and activity subsequently measured at 55°C for 10 minutes.

α- and β-naphthyl phosphate

SsoPTP protein phosphatase activity was assayed against the two isomers of naphthyl phosphate to determine the depth of the active site pocket. The release of inorganic phosphate was measured by the Malachite green assay (Lanzetta *et al.* 1979). Assays were performed in a final volume of 250 µl containing 100 mM MES pH 6.0, 0.5 µg of SsoPTP was mixed with 10

mM of either α- or β-naphthyl phosphate. Assays were initiated by the addition of substrate. The reaction mixture was incubated for 10 minutes at 55°C, following which a 50 µl aliquot of the mixture was immediately added to 800 µl of the color reagent, which also stops the reaction. The color reagent was prepared by mixing 3 volumes of 0.045% (w/v) malachite green solution with 1 volume of 4.2% (w/v) ammonium molybdate solution, and let to stand for 20 minutes. Next, 100 µl of 34% (v/v) citrate solution was added and the solution mixed. A standard curve for 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 nmoles of phosphate using a standard solution of 0.5 mM K_2HPO_4 was prepared. All samples were allowed to stand for 15-30 minutes for ful color development before their OD was measured at 660 nm.

Phosphoprotein substrates

To determine the substrate specificity of SsoPTP *in vitro*, i.e. whether it hydrolyzes phosphotyrosyl residues exclusively or phosphoesters of all the hydroxyl amino acids side chains (phosphotyrosine, phosphothreonine, and phosphoserine), a series of exogenous [^{32}P] phosphoprotein substrates were assayed by the procedure of McGowan and Cohen (McGowan *et al.* 1988). Assays were conducted in a 50 µl reaction volume containing 100 mM MES pH 6.0 and 0.2 mg/ml BSA, 50 ng SsoPTP, and phosphoprotein substrate (Casein, RCML, or MBP) at various concentrations of protein-bound [^{32}P] phosphate (0.1, 0.3, 0.5, 1, 3, 5, or 8 µM). Assays were initiated by the addition of substrate. Following incubation at 55°C for 10 minutes, the reaction was terminated by the addition of 150 µl of 20% (w/v) Trichloroacetic acid (TCA). After agitating briefly on a Vortex mixer, the mixture was centrifuged for 3 minutes at 12, 000 x *g*. A portion, 50 µl, of the supernatant liquid was removed and counted for ^{32}P radioactivity in 1.0 ml of Scintisafe 30% liquid scintillation fluid.

The source of the ^{32}P-labeled material in the TCA supernatant was verified to be inorganic phosphate via extraction of phosphate into an organic phase as a Molybdate complex. Briefly, 75 µl of the TCA supernatant was transferred to a clean 1.5 ml microcentrifuge tube. A 200 µl volume of Molybdate reagent was added, followed by 10 µl of 4 mM K_2HPO_4 (as carrier) and 200 µl of 1:1 isobutanol:toluene. The mixture was agitated vigorously on a Vortex mixer for 30 seconds. The tube was then centrifuged for 3 minutes at 12, 000 x *g* to separate the organic and aqueous phases. A 50 µl portion of the upper, organic layer was removed and placed in a scintillation vial containing 1 ml of liquid scintillation fluid, and was counted for ^{32}P-radioactivity.

Preparation of ^{32}P-labeled-serine-threonine or tyrosine phosphoproteins

The *lyn* protein tyrosine kinase was used to phosphorylate tyrosine residues in casein, RCML (reduced, carboxyamidomethylated, and maleylated lysozyme), and MBP (myelin basic protein) as described in (Tonks *et al.* 1988). Briefly, 100 µl of 5% (w/v) casein solution or 1 mg RCML or MBP was mixed with 500 µl *lyn* kinase which had been immobilized on beads were combined in a 1 ml reaction volume containing 50 mM HEPES pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 0.015% (w/v) Brij-25, 0.2 mM cold ATP, and 900 µCi [γ-^{32}P] ATP, The reaction mixture was rotated on a rotating wheel overnight at room temperature.

The catalytic subunit of cAMP-dependent protein kinase (PKA) was used to phosphorylate the above substrates on serine/threonine residues (McGowan *et al.* 1988). Briefly, a 100 µl of 5% (w/v) casein solution or 2 mg RCML or 1 mg MBP was mixed with 2500 units of PKA were combined in a 500 µl reaction volume containing 1 mM DTT, 0.1 mM EGTA, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.2 mM cold ATP, 900 µCi [γ-^{32}P] ATP. The reaction

mixture was rotated on a rotating wheel overnight at room temperature. The reaction was stopped by the addition of 50 µl of 100 mM EDTA/100 mM sodium pyrophosphate, pH 7.0.

All phosphoprotein, with the exception of RCML, were purified by size exclusion chromatography on a 25 cm, 40 ml column of Sephadex G-25 equilibrated with 2 bed volumes of the equilibration buffer (50 mM Tris-HCl pH 7.5). The phosphotyrosine substrates reaction mixture was centrifuged for 5 minutes at 12, 000 x g to eliminate the beads bearing *lyn* kinase. For both type of reactions, an aliquot of 5 µl of the reaction mixture was transferred to a clean 1.5 ml microcentrifuge tube containing 995 µl of water prior to gel filtration for the purpose of determining the specific radioactivity of the ATP used. The remaining mixture was then applied to the G-25 column and the column developed with equilibration buffer. A total of 15 fractions of 1 ml volume each were collected and aliquots of 5 µl were counted for ^{32}P radioactivity. The first radioactive peak corresponds to the purified substrate.

^{32}P-Y-RCML or ^{32}P-S/T-RCML were separated from [^{32}P]ATP by TCA precipitation. Briefly, 200 µl of 100% (w/v) TCA is added to the reaction mixture and the mixture is then agitated on a Vortex mixer. After standing for 2 minute, the tube was then centrifuged for 5 minutes at 12, 000 x *g*. The supernatant liquid was decanted and the pellet was resuspended with 0.5 M Tris-HCl pH 8.4. The mixture was agitated using a Vortex mixer and rotated on a rotating wheel until the pellet dissolved. The procedure was repeated five times. A 5 µl portion of the reaction mixture was counted for ^{32}P radioactivity to determine the concentration of the radioactive substrate.

To express GST/*lyn* kinase, the following protocol was used (Howell *et al.* 1996). Frozen glycerol stock of *E. coli* carrying the pGEX-KT plasmid was streaked on a 2XYT/ampicillin plate and incubated overnight at 37°C. Isolated colonies were picked and inoculated into four

culture tubes containing 5 ml of liquid 2XYT/amplicillin medium. The cultures were incubated overnight at 37°C with shaking (~200-250 rpm). All four cultures (20 ml total) were used to inoculate 1 liter of 2XYT/ampicillin medium. The culture was then incubated at 37°C with vigorous shaking for 3 hours. Expression of recombinant proteins was induced by adding 200 µl of 1M IPTG to a final concentration of 0.8 mM. The culture was allowed to grow for another 3 hours, after which the cells were harvested by centrifugation and the cell pellet can be stored at -80°C for several weeks.

In order to purify GST/*lyn* kinase, cell pellets were first resuspended in 10 ml PBS buffer (phosphate-buffered saline) containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, and 1x protease inhibitor cocktail. Cells were then lysed by sonic disruption using a large probe. After sonication, Triton X-100 was added to a final concentration of 1% (v/v). The homogenate was centrifuged at 10, 000 x g for 10 minutes at 4°C to remove cell debris and other insoluble material. The supernatant was collected for purification of GST/*lyn* kinase fusion protein. The supernatant was mixed with 1 ml of a 50% (v/v) suspension of glutathione-agarose beads and incubated for 30 minutes at 4°C with gentle shaking or rotation. The glutathione-agarose beads were pelleted in a tabletop centrifuge and the supernatant liquid was discarded. The glutathione-agarose beads were washed at least 4 times by resuspension in 10 ml of ice-cold PBS containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, and protease inhibitors, followed by centrifugation and removal of the supernatant liquid. The duration of each wash step was about 5 minutes. After washing, the glutathione-agarose beads were suspended as a 50% slurry in PBS containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, and protease inhibitors. At this stage, the GST/*lyn* kinase was left on beads. The beads were stored in a 50% (v/v) suspension in glycerol/PBS buffer (1 ml of 80% (v/v) glycerol and 1 ml PBS).

SsoPTP protein variants

Primer design

Primers were designed to contain the desired mutation, flanked by unmodified nucleotide sequences that are complementary to the plasmid DNA template (SsoPTP plasmid). Primers were used to introduce mutations that produced the following amino acid substitutions: Cys^{96} and Gln^{139} to Ser; Asp^{69}, His^{95}, Gln^{135} and Gln^{139} to Ala; Gln^{139} to Glu, Asn, or Asp; and Arg^{102} and Arg^{130} to Lys or Ala. All the primers used in site directed mutagenesis are listed in Table 2-1.

Primer	Direction	Sequence (5'-3')
C96S	Forward	GAAGGTAATCTAGTTCATAGTGTAGGTGGAATAGGAAGGAC
	Reverse	GTCCTTCCTATTCCACCTACACTATGAACTAGATTACCTTC
D69A	Forward	TTACATATTCCAATTCCAGCTGGGGGAGTTCCATCGGATTC
	Reverse	GAATCCGATGGAACTCCCCCAGCTGGAATTGGAATATGTAA
H95A	Forward	AAAGAAGGTAATCTAGTTGCTTGTGTAGGTGGAATAGGAAGG
	Reverse	CCTTCCTATTCCACCTACACAAGCAACTAGATTACCTTCTTT
Q135A	Forward	GTAAGACCTGGTGCAGTAGCAACATATGAGCAAGAGATGTTC
	Reverse	GAACATCTCTTGCTCATATGTTGCTACTGCACCAGGTCTTAC
Q139A	Forward	GCAGTACAAACATATGAGGCAGAGATGTTCTTATTACGCGTG
	Reverse	CACGCGTAATAAGAACATCTCTGCCTCATATGTTTGTACTGC
Q139E	Forward	GCAGTACAAACATATGAGGAAGAGATGTTCTTATTACGCGTG
	Reverse	CACGCGTAATAAGAACATCTCTTCCTCATATGTTTGTACTGC
Q139N	Forward	GCAGTACAAACATATGAGAACGAGATGTTCTTATTACGCGTG
	Reverse	CACGCGTAATAAGAACATCTCGTTCTCATATGTTTGTACTGC
Q139D	Forward	GCAGTACAAACATATGAGGACGAGATGTTCTTATTACGCGTG
	Reverse	CACGCGTAATAAGAACATCTCGTCCTCATATGTTTGTACTGC
Q139S	Forward	GCAGTACAAACATATGAGAGCGAGATGTTCTTATTACGCGTG
	Reverse	CACGCGTAATAAGAACATCTCGCTCTCATATGTTTGTACTGC
R102K	Forward	TGTGTAGGTGGAATAGGAAAGACTGGTACAATTTTAGCTAG
	Reverse	CTAGCTAAAATTGTACCAGTCTTTCCTATTCCACCTACACA
R102A	Forward	TGTGTAGGTGGAATAGGAGCGACTGGTACAATTTTAGCTAG
	Reverse	CTAGCTAAAATTGTACCAGTCGCTCCTATTCCACCTACACA
R130K	Forward	GACGAAGTTAGATTGGTAAAACCTGGTGCAGTACAAACATATG
	Reverse	CATATGTTTGTACTGCACCAGGTTTTACCAATCTAACTTCGTC
R130A	Forward	GACGAAGTTAGATTGGTAGCACCTGGTGCAGTACAAACATATG
	Reverse	CATATGTTTGTACTGCACCAGGTGCTACCAATCTAACTTCGTC

Table 2-1. Primer sets (shown in 5'-3'direction) used in site-directed mutagenesis for generating SsoPTP variants.

Site-directed mutagenesis

Mutations were introduced via PCR by using the QuickChange II site-directed mutagenesis kit according to manufacturer's protocol. Every set of forward and reverse primers, 10 pmoles each, was mixed with 5-50 ng of the appropriate SsoPTP plasmid DNA template in a 50 µl reaction volume containing 5 µl of 10x reaction buffer and 1 µl of 5mM dNTP mix. Then 1 µl of *Pfu Ultra* DNA polymerase (2.5 U/µl) was added to the mixture. PCR was carried out in a thermo cycler programmed with the following parameters: one step at 95°C for 30 seconds, 18 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 1 minute. Once the PCR reaction was complete, 1 µl of *Dpn*I restriction enzyme (20 U/µl) was added. Each reaction mixture was gently and thoroughly mixed, spun down in a microcentrifuge for 1 minute to collect all liquid at the bottom of the tube, and immediately incubated at 37°C for 1 hour to digest the supercoiled DNA template. A portion, 1 µl, of *Dpn*I-treated DNA from each sample reaction was transformed into separate 25 µl aliquots of a slurry containing α-select chemically competent *E. coli*. The protocols for isolating the resulting plasmids, verifying their sequences, and recombinant protein expressions and purifications were as previously described for the wild type (WT) protein. The concentration of each SsoPTP protein variant was determined by Bradford assay. The purity of each protein was assessed on an SDS-PAGE gel as a single band. The activity of each SsoPTP protein variant was assayed towards *p*NPP under conditions similar to those described above for the WT enzyme.

Chemical rescue of catalytic activity of SsoPTP protein variants

SsoPTP wild type and all SsoPTP protein variants were assayed against *p*NPP and rescue agents were added to the reaction mixture at different concentrations in an attempt to restore or

enhance the activity of inactive or catalytically impaired SsoPTP protein variants. To restore the activity of SsoPTP-C96S, 1, 10, 100, 300, 500, or 1000 mM of β-mercaptoethanol was added to reaction mixture. Acetic acid at concentrations of 1, 100, 300, 500, 1000, 2000, or 3000 mM or bicarbonate at 100 or 500 mM were each added to the reaction mixture to restore SsoPTP-D69A activity. For SsoPTP-R102A (K) and SsoPTP-R130A (K), 0.5, 1, 5, 10, 50, 100, 200, 300, or 500 mM Guanidine-HCl was added to the reaction mixture. A concentration of 1, 5, 10, 100, or 200 mM of Imidazole was added to assays of SsoPTP-H95A. Acetamide at a concentration of 10, 50, 100, 200, or 500 mM was used for SsoPTP-Q139A and SsoPTP-Q135A rescue. Each reaction was assayed under similar conditions as those described for the WT enzyme.

Tryptic digest of SsoPTP and its protein variants

To check whether altering each of the following residues: Cys^{96} to Ser; Asp^{69}, His^{95}, Gln^{135} and Gln^{139} to Ala; and Arg^{102} and Arg^{130} to Lys or Ala; caused a conformation change in the protein, partial proteolysis was employed as a probe of protein structure. A 30 µg portion of each protein variant was incubated with 8 µg of trypsin at 37°C for 0, 15, 30, and 60 minutes in a total reaction volume of 250 µl containing 100 mM Tris pH 7.0. A 30 µl aliquot of the digestion mixture was removed, 7.5 µl of 4x SDS loading buffer (200 mM Tris-HCl pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 100mM DTT) immediately added, and the mixture heated at 100°C to stop the reaction at the desired time. Each reaction aliquot was then subjected to SDS-PAGE gel. Each gel was stained with 0.075% (w/v) Coomassie R-250 Brilliant Blue staining for at least 30 minutes, and then de-stained with 10% (v/v) acetic acid and 20% (v/v) methanol for at least 2 hours (Fairbanks *et al.* 1971). Images of the stained gels were recorded with a Bio-Rad imager.

Trapping the phosphocysteine enzyme intermediate

To trap the phosphocysteine enzyme intermediate, several ^{32}P-labeled phosphopeptides or phosphoproteins were incubated with wild type SsoPTP or a protein variant and the reaction was rapidly stopped to try and capture a ^{32}P-C-phophoenzyme intermediate. Briefly, in a 10-50 µl volume containing 100 mM MES, pH 6.0, from 1-55 pmoles (0.02-1µg) of SsoPTP WT, SsoPTP-C96S, or SsoPTP-Q139A was mixed with 5-20 pmoles (5-1000 nM) of each of ^{32}P-Y-Raytide, ^{32}P-Y-Angiotensin I, ^{32}P-S-Kemptide, and ^{32}P-S/T-BSA, ^{32}P-S/T-RCML, ^{32}P-S/T-casein, , and ^{32}P-Y-casein. The reaction(s) was stopped by the addition of 1x SDS loading buffer (50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue) at 0-20 minutes. The reaction(s) was done either at room temperature or 55°C. The samples were subjected to SDS-PAGE without heating followed by autoradiography using a Packard Instant Imager.

Preparation of ^{32}P-Y-Raytide, ^{32}P-Y-Angiotensin I, ^{32}P-S-Kemptide, and ^{32}P-S/T-BSA

Phosphorylation of ^{32}P-Y-Raytide (a 19 residue peptide containing a single tyrosine residue), and ^{32}P-Y-Angiotensin I (a 10 residue peptide containing a single tyrosine residue) was performed under similar conditions to those previously described for preparing ^{32}P-Y-phophoprotein substrates with the exception that the volume of the reaction was reduced to 300 µl and 80 µg of Raytide, or 200 µg of Angiotensin I was used. Phosphorylation of ^{32}P-S-Kemptide (one serine/mole peptide of 7 residues long) and ^{32}P-S/T-BSA was done in a similar fashion as those previously described for ^{32}P-S/T-phosphoproteins with the exception that the reaction volume of the ^{32}P-S-Kemptide was reduced to 50 µl. About 20 µg of Kemptide and 1 mg of BSA were used. ^{32}P-S/T-BSA was purified by size exclusion chromatography as

previously described. To purify the ^{32}P-labeled phophopeptides, a 50 µl aliquot of the reaction mixture was spotted on a 2x2 cm P81 phosphocellulose paper to a total of 4 papers (200 µl reaction volume). The papers were placed in a metal basket, made of galvanized steel mesh, placed in a 600 ml beaker containing 250 ml of 150 mM phosphoric acid (H_3PO_4) and agitated for 20 min with a stir bar. The liquid was then discarded and the process repeated three times more. To elute bound peptides, the papers were placed in a 2 ml tube and 1 ml of 50 mM ammonium bicarbonate was added. The free liquid was collected and the process repeated once more. The resulting eluates were pooled and lyophilized using a SpeedVac concentrator. The resulting pellets were resuspended with 300 µl of 20 mM Tris-HCl pH 7.0. An aliquot, 5 µl, of the suspension mixture was transferred to 1 ml of scintillation fluid and counted for ^{32}P radioactivity.

Identification of endogenous SsoPTP substrates by substrate trapping

Cultures of *S. solfataricus*

Sulfolobus *solfataricus* P2 ATCC strain 35091 was grown in liquid medium consisting of: 1 g/L of yeast extract, 1 g/L of casamino acids, 3.1 g/L KH_2PO_4, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.2H_2O$, and 0.25 g/L $CaCl_2.2H_2O$ (de Rosa *et al.* 1975). The pH of the medium was adjusted to 2.5-3.0 with sulfuric acid. The medium was autoclaved, then 10 ml of 100x trace elements solution added. The 100X trace elements solution consists of 0.180 g/L $MnCl_2.4H_2O$, 0.450 g/L $Na_2B_4O_7.7H_2O$, 0.022 g/L $ZnSO_4.7H_2O$, 0.005 g/L $CaCl_2.2H_2O$, 0.003 g/L $Na_2MoO_4.2H_2O$, 0.003 g/L $VOSO_4.3H_2O$, and 0.001 g/L $CoSO_4$. The pH of the trace elements solution is adjusted to 4.0 with H_2SO_4 and the solution is autoclaved.

First, *S. solfataricus* P2 was grown in a 10 ml volume of medium to which 50 µg/ml of kanamycin had been added, then incubated at 72°C for 3 to 5 days with vigorous shaking (~100-200 rpm). A 5 ml volume of the actively growing culture was used to inoculate a 250 ml volume of fresh media containing 50 µg/ml of Kanamycin. The culture was left to grow for 3 to 5 days until the OD_{540} fell within the range of 0.6-1.0. The cells were then harvested by centrifugation for 15 minutes at 3000 x *g*. The pellets were stored at -80°C; the cell pellets can be left for several weeks before processing. To store *S. solfataricus* P2 for long periods (several years), a small pellet from the 10 ml culture was resuspended in 930 µl of fresh medium. DMSO, 70 µl, was then added and the suspension was flash frozen in a dry ice / ethanol bath and stored at -80°C.

Preparation of *S. solfataricus* cell lysates

S. solfataricus was cultured in 4 L of media until the OD_{540} fell in the range of 0.9-1.0 (about 5 days). To inhibit endogenous protein phosphatases, pervanadate then was added to a final concentration of 1 mM and the culture incubated for an additional 30 minutes (Blanchetot *et al.* 2005). Pervanadate was freshly prepared by mixing equal volumes of 200 mM sodium orthovanadate with 200 mM hydrogen peroxide. The cells were then harvested by centrifugation for 15 minutes at 3000 x *g*. The pellets of about 8 g wet weight were suspended in suspension buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton-X100, 10% (v/v) glycerol, 5 mM iodoacetic acid, and 1x protease inhibitors cocktail (100 mM AEBSF, 5 mM Bestain, 1.5 mM E-64, 2 mM Pepstatin A, and 0.2 mM phosphoramidon). The suspension buffer contained 5 mM iodoacetic acid to irreversibly inactivate endogenous PTPs, and EDTA to chelate and inactivate divalent metal ion dependent protein-serine/threonine phosphatases as well as any metalloproteases. The cells were lysed by sonic disruption using a

W185 sonicator set at 60 watts. The crude cell lysate was kept on ice for 20 minutes. DTT was added to the crude cell lysate to a final concentration of 10 mM to consume any unreacted iodoacetic acid and pervanadate. The cell lysate was kept at 4°C for 15 minutes. Using a Beckman ultracentrifuge, the cell lyaste was centrifuged at 40, 000 x g for 30 minutes at 4°C.

Coupling of enzyme to NHS-activated Sepharose 4 fast flow

The wild type enzyme and two SsoPTP protein variants: SsoPTP-C96S/D69A and SsoPTP-Q139A/D69A were used to trap any possible endogenous phosphoprotein substrates that might be present in the lysates of *S. solfataricus*. The plasmids encoding the two protein variants were generated using the QuickChange II site-directed mutagenesis kit. To construct SsoPTP-C96S/D69A or SsoPTP-Q139A/D69A plasmids, the previously designed primers for SsoPTP-D69A were used with the previously isolated plasmids of SsoPTP-C96S or SsoPTP-Q139A as templates. The two protein variants were expressed, and purified as previously described for the wild type enzyme.

The wild type enzyme and the two protein variants were coupled to NHS (N-hydroxysuccinimide)-activated Sepharose beads according to manufacturer's protocol. First, a 2 ml bed volume of beads was washed with 10 ml 1 mM ice-cold HCl. The slurry was rotated for 5 minutes, and centrifuged at 500 x *g* for 2 minutes and the supernatant liquid discarded. This process was repeated once. To prepare the coupling solution, 10 mg of protein (WT or SsoPTP-C96S/D69A or SsoPTP-Q139A/D69A) was added to 5 ml of coupling buffer (100 mM MES pH 6.0). The coupling solution and the HCl-washed beads were combined and incubated overnight at 4°C with constant agitation using a rotating wheel. The reaction mixture was then centrifuged for 3 minutes at 500 x *g*. The resulting supernatant was saved for analysis. After the coupling

was completed, any non-reacted groups on the resin were blocked by adding 10 ml of 100 mM Tris-HCl pH 8.5 for 3 hours at 4°C with rotation. The mixture was then centrifuged for 3 minutes at 500 x *g*. The resin was washed with two alternating buffers, 100 mM Tris pH 8.5 and 100 mM acetate pH 4.0, 500 mM NaCl. Each washing step consisted of a 10 ml of buffer added to the beads, rotating the mixture for 5 minutes, centrifuging for 3 minutes at 500 x *g*, and discarding the supernatant liquid. This cycle was repeated 4 times. The coupled affinity resin was then resuspended in 2 ml of 100 mM MES pH 6.0. Coupling efficiency was assessed by calculating the difference between the protein added and that recovered in the supernatant after incubation using Bradford assay. The catalytic competency of the immobilized WT enzyme was assessed by incubating a portion of the beads with *p*NPP at 55°C for several minutes.

Binding and elution of *S. solfataricus* proteins

A volume of 7 µl of cell lysate from *S. solfataricus* was added to 2 ml of each NHS-activated Sepharose-bound WT or protein variants and left at 4°C overnight with constant mixing rotation on a rotating wheel. After incubation, the reaction mixture was centrifuged for 3 minutes at 500 x *g*. The resulting supernatant was saved for SDS-PAGE analysis. The beads were washed three times in 10 ml of the suspension buffer without iodoacetic acid and protease inhibitors cocktail (buffer A). For each wash, the beads were centrifuged for 3 minutes at 500 x *g*. The resulting supernatant was decanted. The washed beads were then placed in a filter column. The column was washed with 10 ml of buffer A. To elute *S. solfataricus* proteins that were bound to the column, 2 ml of buffer A containing 0.5M NaCl was used, followed by 4 ml of buffer A containing 1 M NaCl, 2 ml volume of buffer A containing 1.5 M NaCl, and finally 2 ml of buffer A containing 2 M NaCl. The eluant liquid was collected in 1 ml fractions.

To visualize the eluted proteins on an SDS-PAGE gel, samples were first concentrated by TCA precipitation. In a clean 1.5 ml Eppendorff tube, 1 ml of 10% (w/v) TCA was added to 400 µl sample. The tube(s) was then inverted to mix and placed on ice for 10 minutes. The tube was spun down at 12, 000 x *g* for 10 minutes at 4°C. The supernatant was decanted by gentle pipetting. The resulting pellet was washed gently with 500 µl of 1% (w/v) TCA to remove access TCA. The tube was centrifuged at 12, 000 x *g* for 5 minutes at 4°C. The supernatant was decanted by pipetting. The pellet was resuspended with 50 µl 1x SDS loading buffer, centrifuged, and heated at 100°C for 5 minutes. Samples were then subjected to SDS-PAGE. The gel(s) was either stained with Coomassie blue or transferred to a membrane for Western blot analysis. Several bands from the gel were excised for analysis by mass spectroscopy.

Mass spectral analysis

Sections of acrylamide gels containing bands of interest were excised and chopped into approximately 1 mm^3 pieces using a clean razor blade. In order to remove the Coomassie stain, pieces were transferred into microfuge tubes and 25 mM ammonium bicarbonate : acetonitrile (1:1) was added at approximately 20x the volume of the gel pieces. The microfuge tubes containing the gel pieces were vortexed vigorously for 2 hours. The free liquid was removed using a pipette and the process was repeated one more time. Next, ten gel volumes of acetonitrile was added to the gel pieces. After 15 minutes, the acetonitrile was removed by pipetting. Gel pieces were then rehydrated on ice using just enough 10 ng/µl trypsin in 25 mM ammonium bicarbonate to cover the gel pieces. The digestion mixture was then incubated overnight (typically 16-18 hours) at 37ºC.

The next day the mixtures were centrifuged briefly to collect liquid at the bottom of the tubes, and the solution transferred to a new microfuge tube that had been rinsed with acetonitrile. Next, to increase peptide recovery, 50% acetonitrile supplemented with 0.2% trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated in a water bath for approximately 15 minutes. The free liquid was then removed, combined with the first and mixed thoroughly. When necessary, digests were desalted and concentrated using OMIX C18 microextraction pipette tips (Varian) following the manufacturer's protocol prior to MALDI analysis.

Approximately 1 µl of each digest was spotted onto a MALDI target plate and allowed to air dry. Each dried "spot" was then overlaid with approximately 1 µl of matrix solution. The matrix solution used was 4 mg/ml α-cyano-4-hydroxy cinnamic acid in 50% (v/v) acetonitrile containing 0.2% (v/v) TFA and 20 mM ammonium citrate.

The digests were then analyzed using a 4800 MALDI Tof/Tof mass spectrometer (Applied Biosystems). Initial MS spectrum was collected for each digest in reflector positive operating mode for the mass to charge range of 800 to 4000. Each spectrum was the cumulative product of approximately 1000 individual laser shots. Next, MSMS data was collected for the top 16 peaks from each digest above a set signal to noise threshold using the MSMS 1kV positive operating mode. Each MSMS spectrum was typically the sum of approximately 1500 individual laser shots. A peak list for each digest containing information from both the MS and MSMS spectra was generated using the 4000 Explorer (Applied Biosystems) software.

Protein identifications were obtained by searching the peak lists versus the NCBI nonredundant protein database (version 20100226) limited to archaebacterial proteins using the web-based Mascot search engine (Matrix Science). The following search parameters were

employed: trypsin protease specificity with up to one missed cleavages, peptide mass tolerance ±150 ppm, peptide fragment mass tolerance ±0.15Da with the possible modification of methionine by oxidation.

Protein identifications were considered significant if at least two unique peptides from the protein with individual ion scores corresponding to $p<0.05$ were identified from the MSMS data.

SsoHSP20 and SsoATPase from *S. solfataricus*

Cloning, expression, and purification

SsoHSP20 and SsoATPase were identified by mass spectral analysis of the bands excised from the SDS-PAGE gel of the substrate trapping experiment outlined above. To test whether they can form a complex with SsoPTP enzyme, an *in vitro* experiment was attempted. The two proteins were cloned in *E. coli*, expressed, and purified as described above for wild type SsoPTP with the exception that the PCR parameters were as follows: one step at 95°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and one cycle at 72°C for 5 minutes. The forward and reverse primers used to amplify SsoHSP20 and SsoATPase genes for cloning into pET21-d had 5'-tails similar to the primers used for cloning WT SsoPTP gene into pET21-d (*Nco*I and *Sal*I restriction enzyme sites (underlined) were engineered in the 5'-tails). The primers are listed below:

SsoHSP20 forward primer: 5′-ACCCATGGCTATGCCCAAGAGGGAAGAAAAG-3′

SsoHSP20 reverse primer: 5′-AGGAAAGTCGACCTCAACTTTTATGTCAACACCAG-3′

SsoATPase forward primer: 5′-TTCCATGGGCTTGTCAACTTCCCAATCTC-3'

SsoATPase reverse primer: 5′-TTATATTGTCGACACCAATCACCGATCTC-3′

Native gel electrophoresis

SsoPTP, SsoHSP20, and SsoATPase were subject to native gel electrophoresis under nondenaturing conditions designed to permit detection of any protein-protein complexes present. In a 20 µl volume containing 100 mM MES pH 6, 2-10 µg of SsoPTP was mixed with 2-10 µg of SsoHSP20 or SsoATPase. Control incubations contained each protein alone. Incubation was performed either at room temperature for 30 minutes or 55°C for 10 minutes. Samples were prepared by adding 1x native loading buffer (50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue), then loaded onto a 12% native gel. Electrophoresis was carried out at 35 mAmps for 1 hour in running buffer (25 mM Tris and 192 mM glycine). The gel was then stained with Coomassie blue and destained. An image of the gel was captured using a Bio-Rad imager.

Gel filtration chromatography

A second approach for detecting protein complex formation was gel filtration chromatography. A column of Sephadex 200 10/300 GL (bed dimensions 10x300 mm and bed volume 24 ml) was calibrated with macromolecules of known mass (cytochrome c, Vitamin B_{12}, Blue dextran, albumin, carbonic anhydrase, alcohol dehydrogenase, and myoglobin). The column buffer was 50 mM MES pH 6 containing 150 mM NaCl. The void volume of the column was determined by injecting 1 mg/ml blue dextran (200 kDa) that had been dissolved in the column running buffer. Protein calibration standards were run in two sets of four proteins each. Each individual protein was dissolved at 10 mg/ml concentration, and then equal volumes of each protein were combined. Before injection, the protein mixtures were centrifuged for three minutes in a microcentrifuge at 13, 000 x *g* to sediment any insoluble material. A volume of 250 µl of the protein standards mix was injected into the FPLC apparatus. A graph of the elution

volume for each protein was determined using which a standard curve was calculated. Next, SsoPTP or SsoHSP20 was injected into the column separately to determine the elution volume for each protein. Finally, a mixture containing equal concentrations of SsoPTP and SsoHSP20 was injected into the column at a rate of 0.5 ml/min to determine any differences in elution volume that would indicate a complex formation. By using the standard curve of protein standards, the molecular weight of SsoPTP, SsoHSP20 or any complex formation was calculated.

General procedures

Bradford assay

The Bradford Assay was routinely used to estimate protein concentration (Bradford 1976) using a premixed solution of BSA (Pierce, Rockford, IL) as standard.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (Laemmli 1970) on 12% or 15% gels. Protein samples were prepared by adding a final protein to 4x-SDS loading buffer (200 mM Tris-HCl pH 6.8, 8% (w/v) SDS (electrophoresis grade), 0.4% (w/v) bromophenol blue, 40% (v/v) glycerol, and 400 mM DTT (Sambrook 1989)) in a 4:1 ratio. Samples were heated at 100°C for 5 minutes, briefly spun down, and loaded to the wells of the stacking gel along with pre-stained protein standards. Electrophoresis was carried out at 35 mAmps for 1 hour. Gels were stained with 0.075% (w/v) Coomassie R-250 Brilliant Blue for at least 30 minutes. The gel was then placed in destaining

solution of 10% (v/v) acetic acid, 20% (v/v) methanol (Fairbanks et. al., 1971). An image of the gel was captured using a Bio-Rad imager.

Native gel electrophoresis

Native gel electrophoresis was conducted similarly to SDS-PAGE with the exception that SDS is not added to the gels, loading buffer, or running buffer, and protein samples are not heated.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al. (1989).

Western blot analyses

The membrane (Immobilon-P) used for Western blot analyses is a polyvinylidene fluoride (PVDF) microporous hydrophobic membrane for binding proteins that are transferred from a variety of gel matrices. Following SDS-PAGE, gels were electroblotted using a wet tank transfer system according to manufacturer's protocol (Millipore). Briefly, gels were immersed in transfer buffer (1x CAPS, 10% (v/v) methanol) and for 15 minutes. A piece of Immobilon-P membrane was cut to match the dimensions of the gel, and notched on one corner for later orientation. The membrane next was wetted in 100% methanol for 15 seconds, and then immersed in E-pure water for 2 minutes. Lastly, the membrane was immersed for at least 5 minutes in the transfer buffer. The transfer stack was assembled by placing a foam pad on one side of the cassette holder, followed by placing 4 or 6 filter papers on top of the pad. On the other side of the cassette holder, another foam pad was placed. The cassette holder was closed and immersed in transfer buffer so that the transfer stack is wet. The cassette holder was opened and a gel was placed on top of 2 or 3 filter papers, then the sheet of the Immobilon-P membrane was

put on top of the gel, followed by the rest of the filter papers. The cassette holder was closed, and placed in the tank blotting apparatus containing the transfer buffer and a stir bar so that the side of the cassette holder with the gel is facing the cathode (-). The system was run at 4°C for 1 hour at 100 volts (1 Amps). Once the electrotransfer was completed, the membrane was dried by immersing in 100% methanol for 20 seconds, and then placed on a piece of paper towel to let air dry overnight at room temperature.

Immunodetection on blotted PVDF membranes was performed by incubating the blot for 1 hour with 10 ml of a primary antibody (anti-phosphotyrosine antibody from mouse) diluted to 1:1000 (v/v) dilutions in TBS-T buffer that contains 10 mM Tris pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, and 1% (w/v) BSA. The membrane was washed twice in TBS buffer for 10 seconds each, then incubated for 30 minutes with 10 ml of a secondary antibody (goat anti-mouse IgG (whole molecule)-alakaline phosphatase antigen specific antibody) diluted in TBS-T buffer to a 1:2000 (v/v) dilution. The blot was then washed twice in TBS for 10 seconds each. Bromochloroindolyl phosphate (BCIP) was prepared by dissolving one tablet in 10 ml E-pure water. The membrane was incubated in this solution until it was judged that sufficient quantities of the precipitated product had accumulated. The blot was then washed with water to remove the BCIP and stop the reaction. An image of the blot was captured using a Bio-Rad imager. The blotted membrane was then air-dried and stored in a dark place.

Calculation of kinetic parameters

Kinetic analyses were performed with SsoPTP using selected substrates. Michaelis constants (K_m, V_m, k_{cat}, and k_{cat}/K_m) for SsoPTP were determined using *p*NPP, ^{32}P-S/T-casein, ^{32}P-S/T-RCML, and ^{32}P-S/T-MBP as substrates. The concentrations of phosphoproteins

substrates ranged between 0.1 and 8 µM protein-bound ^{32}P, and the concentration of *p*NPP ranged between 1 and 25 mM. Kinetic constants were extrapolated by linear regression analysis of Hanes-Woolf plot. Briefly, a curve of velocity (v), nmoles of ^{32}P release per minute per mg of enzyme, versus substrate concentrations [S], was obtained. A plot of [S]/v versus [S] was then extrapolated and linear regression line was obtained. V_m (nmol.min^{-1}.mg^{-1}) was calculated from the inverse of the slope, whereas K_m (µM) was calculated by multiplying the y-intercept and the calculated value of V_m. The kinetic parameter, k_{cat} (min^{-1}), was calculated from the nmoles of ^{32}P release per min per nmoles of enzyme.

CHAPTER III

Cloning, expression, purification, and characterization of recombinant protein tyrosine phosphatase from *Sulfolobus solfataricus*

The genome of *S. solfataricus* contains ORF *sso2453* that encodes a deduced protein tyrosine phosphatase. This ORF was amplified via PCR, and cloned into a vector that encodes a C-terminal hexahistidine tag to ease purification. The recombinant fusion protein, SsoPTP, was purified using immobilized metal ion affinity chromatography (IMAC). The catalytic activity of SsoPTP was assayed toward *p*NPP. Phosphohydrolase activity of SsoPTP was optimized by using *p*NPP as a substrate in terms of temperature, pH, time, enzyme concentration, and ionic strength effect.

Cloning of *S. solfataricus* ORF *sso2453* into pET-21d vector

ORF *sso2453* encoding a deduced protein tyrosine phosphatase, SsoPTP, from *S. solfataricus* was amplified *via* PCR using *S. solfataricus* genomic DNA as template and two oligonucleotide primers designed to be in-frame with the pET-21d expression vector as shown in Figure 3-1. The PCR products, which were of the predicted size of 486 bp, were purified and then digested with two restriction enzymes, *NcoI* and *SalI*, as described in Materials and Methods. The digested PCR products were purified, and ligated into a pET-21d vector encoding C-terminal hexahistidine fusion domain. The expression vector pET-21d containing ORF *sso2453* was used to transform chemically competent α-select *E. coli* cells. The resulting plasmid, named sso2453, was isolated from 5 ml of overnight cultures of α-select *E. coli* cells using QIAPrep Miniprep Kits as described in Materials and Methods. The correct sequence and

orientation of the gene encoding the full length SsoPTP were verified by sequencing in the forward direction (5'- 3') using the T7 promoter primer and in the reverse direction (3'– 5') using T7 terminator primer as shown in Figure 3-2.

Figure 3-1. ORF *sso2453* and the amino acid sequence of its protein product, SsoPTP. Two forward and reverse primers were designed to introduce recognition sites (underlined) for two restriction enzymes, *NcoI* and *SalI*, respectively. The stop codon, shown in red, is replaced by the *SalI* site in the reverse primer so that the resulting PCR product will be inserted in frame with the vector sequence for the His tag. The *NcoI* site upstream of the start codon is shown in red, is constructed so that the resulting PCR product is in-frame with the pET-21d vector as shown in Figure 3-2.

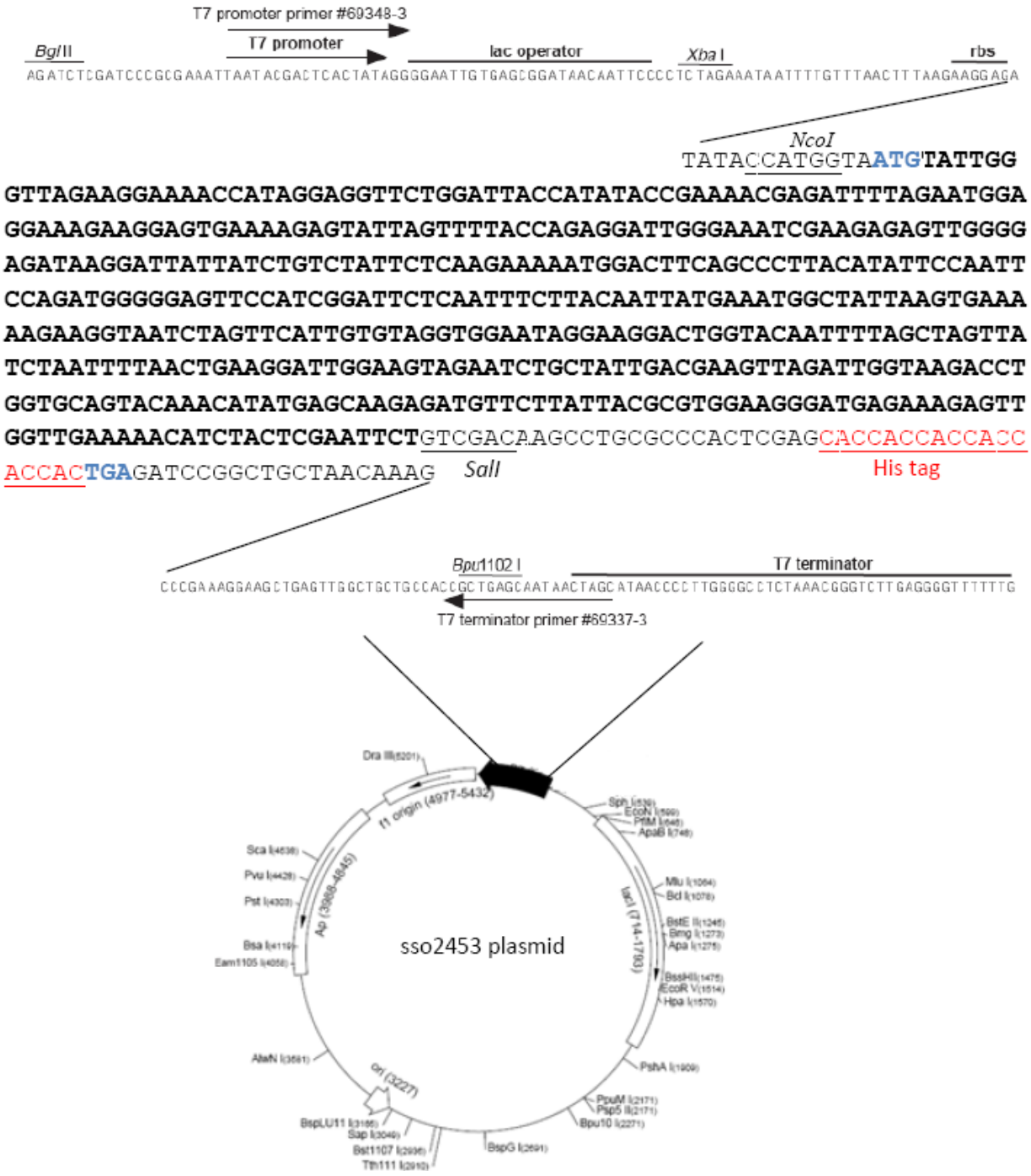


Figure 3-2. Structure of sso2453 plamid. Plasmid sso2453 was constructed by inserting the PCR-generated *sso2453* DNA sequence into a pET-21d vector for the expression of *S. solfataricus* protein, SsoPTP, in *E. coli*. The two recognition sites for *NcoI* and *SalI* are

underlined. The DNA sequence encoding the hexahistidine tag is shown in red and underlined. The start codon of the gene encoding SsoPTP is shown in blue. The stop codon, shown in blue, is placed downstream of the hexahistidine tag in the vector. Part of this image was adapted from Novagen manual for the pET-21d expression system.

Expression of recombinant protein SsoPTP in *E. coli*

Plasmid sso2453 was used to chemically transform *E. coli* BL21-CodonPlus (DE3)-RIL cells. BL21-CodonPlus (DE3)-RIL cells utilize the T7 RNA polymerase IPTG-inducible promoter for high-level protein expression. These cells are engineered to contain extra copies of the *argU*, *ileY*, and *leuW* genes, which encode tRNAs that base pair with the arginine codons, AGA and AGG, the isoleucine codon, AUA, and the leucine codon, CUA, respectively. The extra copies of these tRNA genes allow efficient translation of heterologous proteins in *E. coli* whose genes contain a high proportion of "rare" codons. After 3 hours of growth at 37°C, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to induce SsoPTP expression. The culture was incubated for an additional 3 hours at 37°C before cells were harvested by centrifugation as described in Materials and Methods.

Purification of recombinant fusion protein SsoPTP by IMAC

The recombinant fusion protein, SsoPTP, with C-terminal hexahistidine tag was purified by immobilized metal-affinity chromatography (IMAC). The matrix used for this purification was charged with the transition metal ion, Ni^{2+}, which interacts with the side chains of histidine residues on proteins, and in this case, the hexahistidine tag on recombinant SsoPTP. To help eliminate *E. coli* proteins, cell lysates containing SsoPTP were heated at 55 °C for 10 minutes to denature endogenous proteins. Centrifugation at 10, 000 x *g* for 10 minutes was performed to remove the protein precipitate. SsoPTP, a thermostable enzyme, remained in the soluble fraction, which then was applied to the Ni^{2+} column. SsoPTP bound to Ni^{2+} metal ions in the column and was subsequently eluted with a buffer containing 500 mM imidazole, which competes with the

hexahistidine tag for Ni^{2+} binding. To remove excess imidazole, the eluent liquid was dialyzed against 20mM Tris pH 7. The concentration of protein was determined by Bradford assay (Bradford 1976) as described in Material and Methods. The purity of the recombinant protein was ascertained by SDS-PAGE gel, which revealed a single band of the predicted M_r, as shown in Figure 3-3.

Characterization of SsoPTP

Purified SsoPTP was assayed for phosphomonesterase activity with a common synthetic substrate for phosphohydrolases, *para*-nitrophenyl phosphate (*p*NPP). SsoPTP displayed phosphohydrolase activity towards *p*NPP to a specific activity of 15000 $nmol.min^{-1}.mg^{-1}$. This compound, depicted in Figure 3-4, was used to assess the optimal activity range for SsoPTP under various conditions of temperature, pH, time, ionic strength, and enzyme concentration as described in Materials and Methods (Figure 3-5).

SsoPTP displayed highest activity at 65°C (Figure 3-5, A); however, preincubating the enzyme at various temperatures for 30 minutes prior to assaying its activity with *p*NPP revealed that SsoPTP was stable (retained its original catalytic activity) at a maximum temperature of 55°C whereas its activity decreased with increasing temperatures (Figure 3-5, B). Therefore, all assays were performed at 55°C.

SsoPTP displayed optimal phosphatase activity at pH 6 (Figure 3-5, C). The catalytic activity of the enzyme decreased below pH 5 and above pH 7. All assays were performed with

100mM MES buffer (4-Morpholineethanesulfonic acid with pka of 6.15) since it has a good buffering capacity at pH 5-7.

The activity of SsoPTP was sensitive to the ionic strength in the reaction solution (Figure 3-5, D). At salt concentrations of 100 mM and above, the catalytic activity of SsoPTP decreased.

The amount of SsoPTP used for assaying against low molecular weight substrates, and phosphoprotein substrates was 0.5 µg and 0.05µg, respectively, for a period of 10 minutes. Those amounts were in the linear range of the assay (Figure 3-5, E and F), during which about 10% of the substrate was consumed.

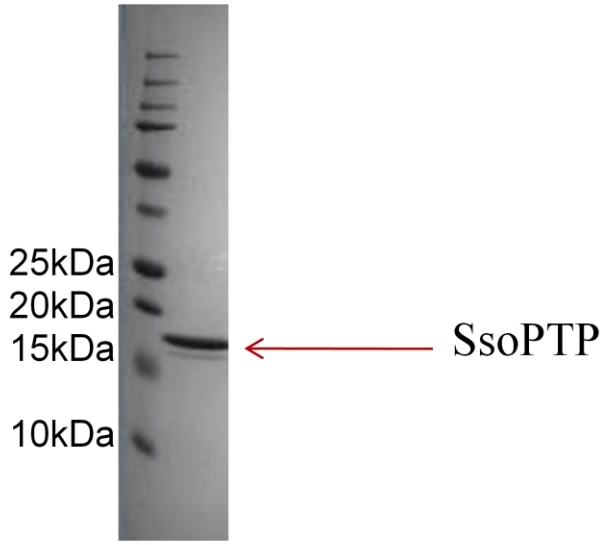


Figure 3-3. Purifed SsoPTP on 12% SDS-PAGE gel. An image of Coomassie-stained 12% SDS-PAGE gel of SsoPTP purified using IMAC. The red arrow indicates a band of size 18kDa, which is the expected size of SsoPTP. The molecular weight standards for SDS-PAGE are shown on the left lane.

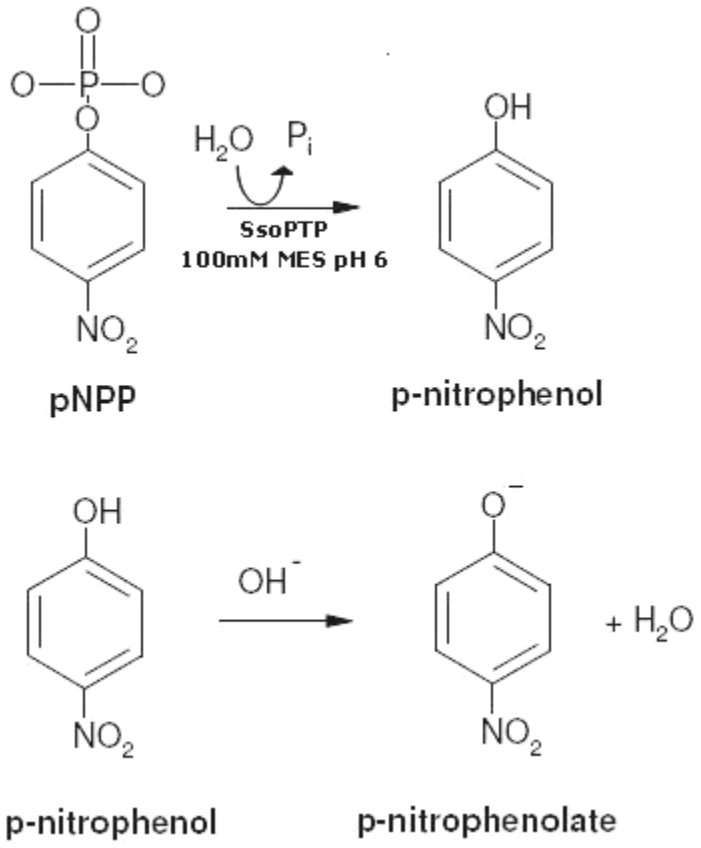
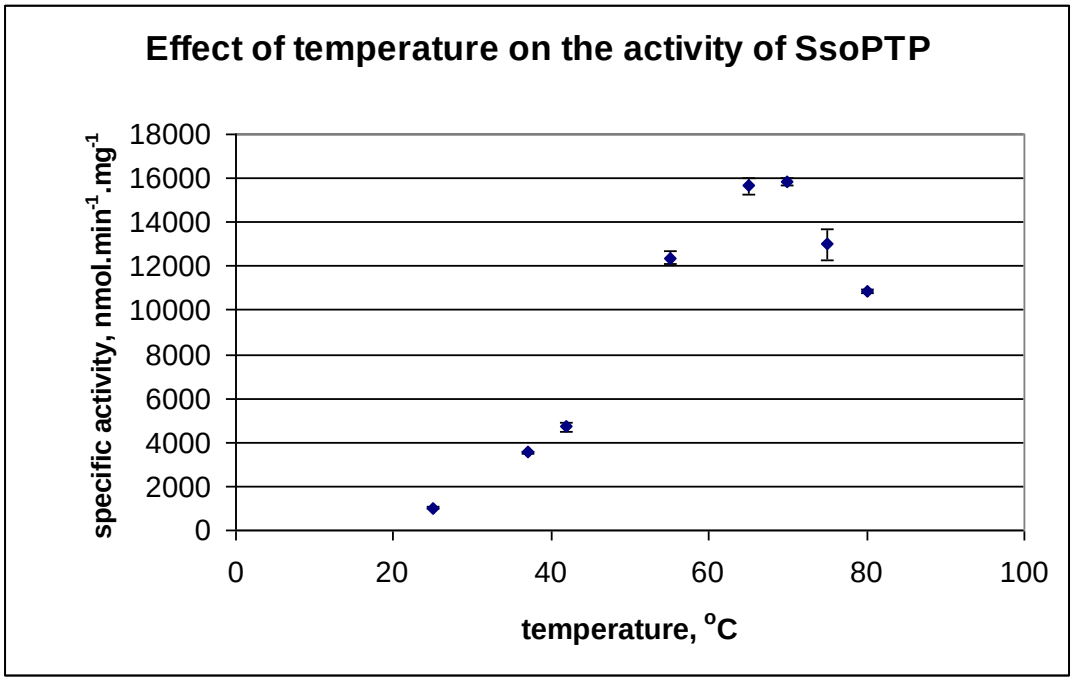
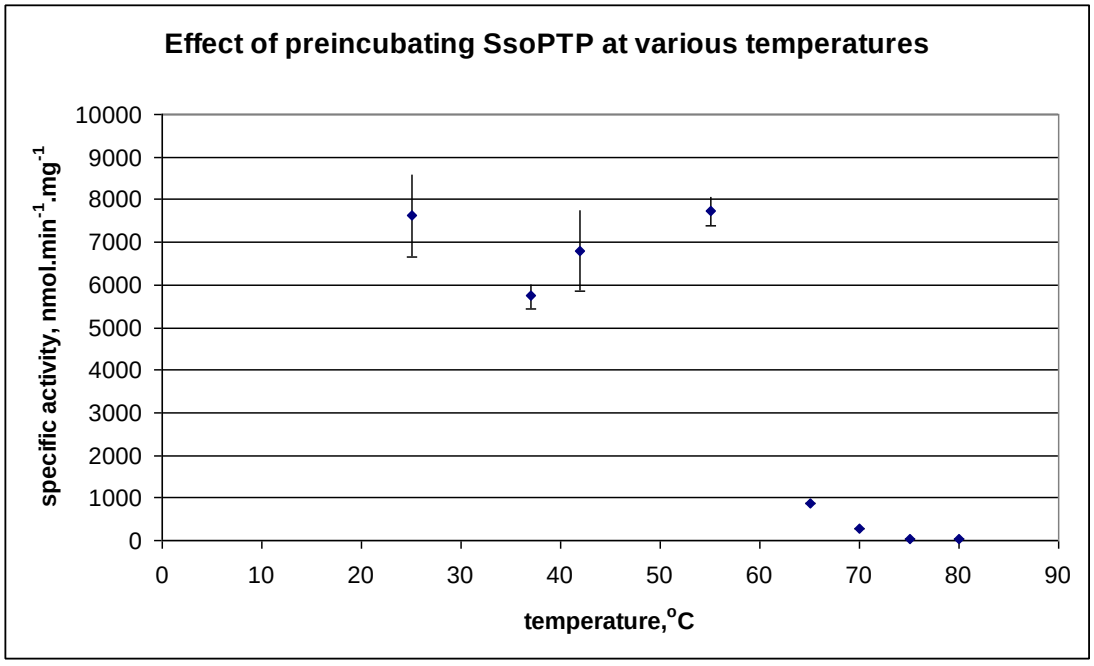


Figure 3-4. SsoPTP phosphatase assay using *p*NPP as a substrate. In the first step, SsoPTP dephosphorylates *p*NPP. In the second step, the OH-group of *p*-nitrophenol is deprotonated under alkaline conditions resulting in *p*-nitrophenolate with a strong absorption at 410 nm (yellow). Adapted from Jena Bioscience Manual for *p*NPP-based colorimetric assays.

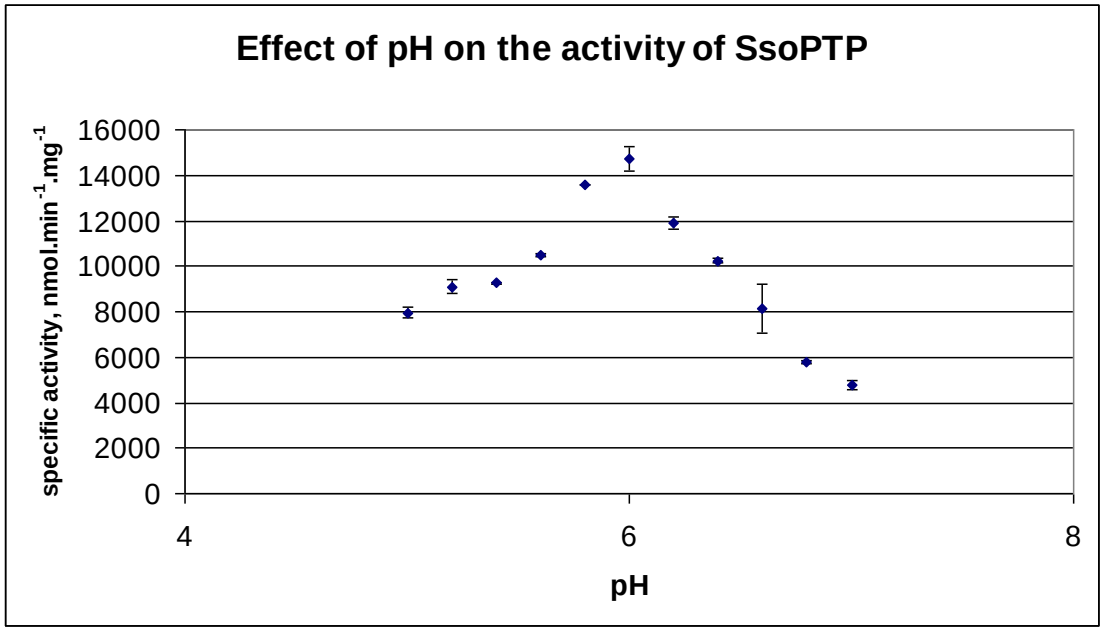
A.


Effect of temperature on the activity of SsoPTP
specific activity, nmol.min-1.mg-1
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
temperature, oC

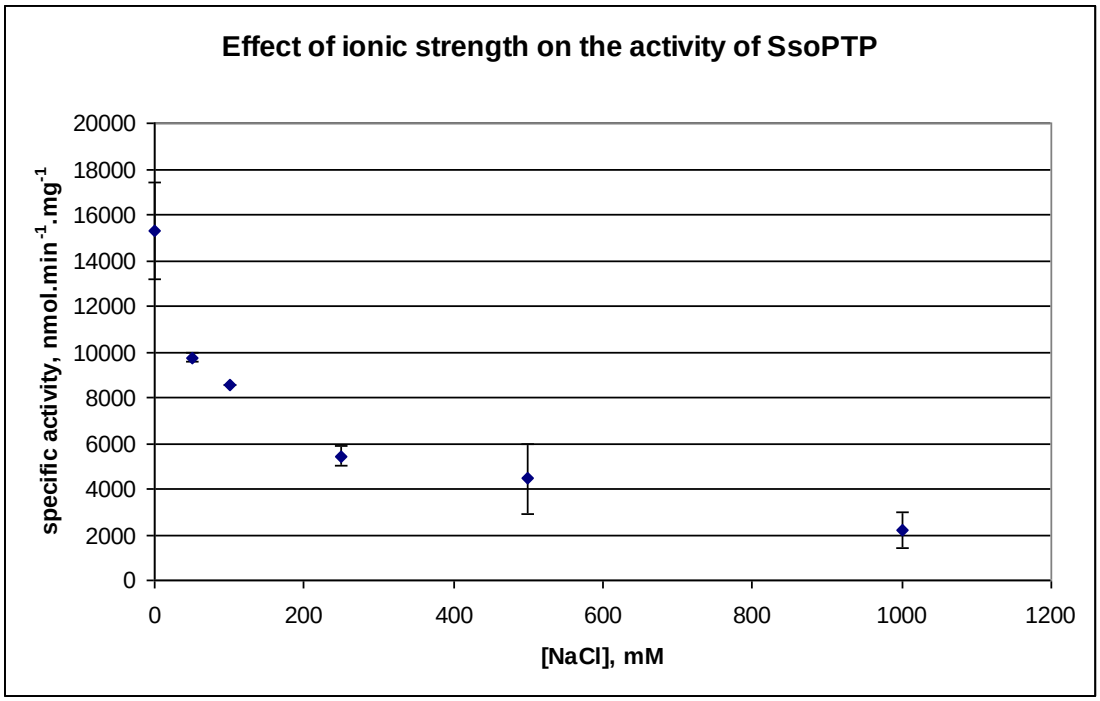
B.


Effect of preincubating SsoPTP at various temperatures
specific activity, nmol.min-1.mg-1
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
temperature,oC

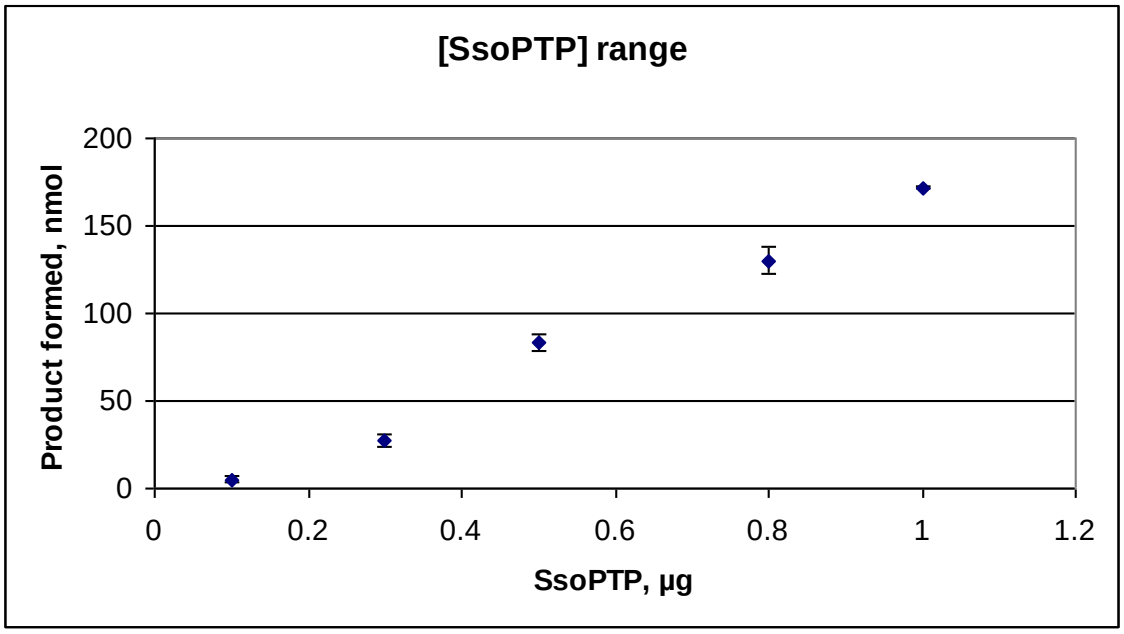
C.


Effect of pH on the activity of SsoPTP
specific activity, nmol.min-1.mg-1
16000
14000
12000
10000
8000
6000
4000
2000
0
4
6
8
pH

D.


Effect of ionic strength on the activity of SsoPTP
specific activity, nmol.min-1.mg-1
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
200
400
600
800
1000
1200
[NaCl], mM

E.



F.

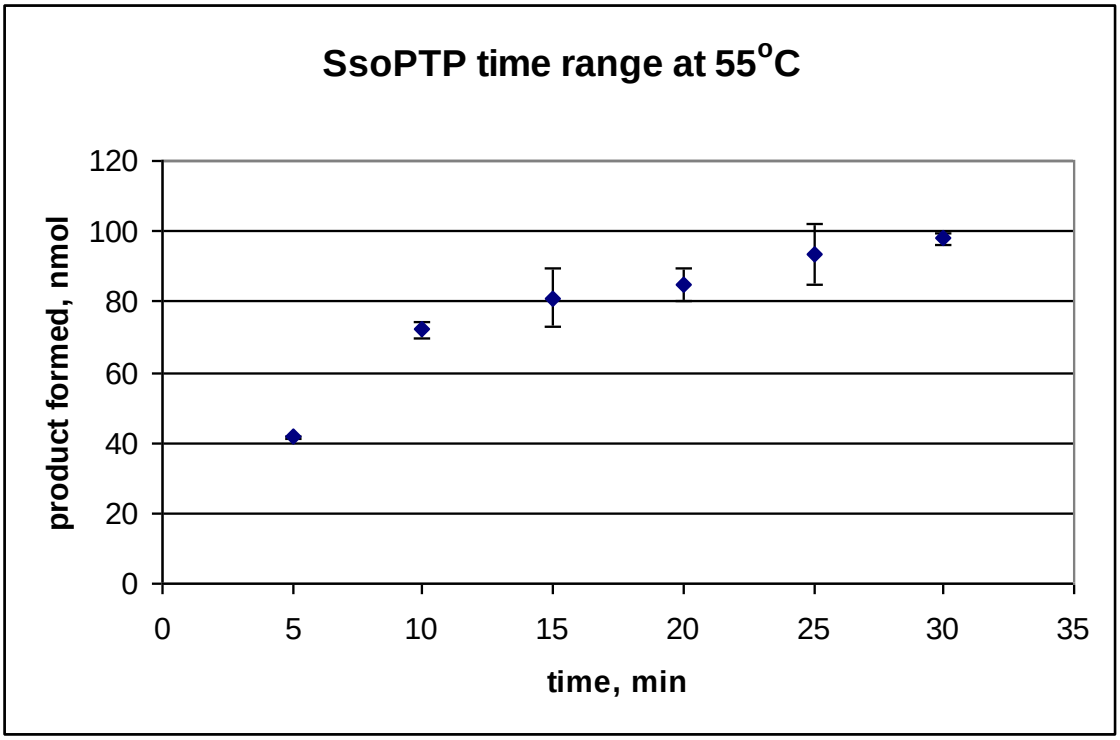


Figure 3-5. Effect of various assay conditions on the catalytic activity of SsoPTP. All assays were performed with 10 mM *p*NPP as a substrate. (A) Effect of temperature on the

catalytic activity of SsoPTP; (B) Effect of preincubating SsoPTP for 30 minutes at various temperatures, then assessing its catalytic activity at 55°C for 10 minutes; (C) Effect of pH on the catalytic activity of SsoPTP with MES buffer (MES has a good buffering capacity range of pH 5-7); (D) Effect of ionic strength on the activity of SsoPTP; (E) Effect of increasing the amount of SsoPTP on its activity in the assay; (F) Effect of increasing the time on the catalytic activity of SsoPTP. All assays were performed three times in duplicates.

CHAPTER IV

Determination of substrate specificity of SsoPTP *in vitro*

The substrate specificity of SsoPTP i.e., whether it hydrolyzes phosphotyrosyl residues exclusively or phosphoesters of all the hydroxyl amino acids (phosphotyrosine, phosphothreonine, and phosphoserine) was determined *in vitro*. Two complementary approaches were employed: exogenous [^{32}P] phosphoproteins and comparison of low molecular weight substrates of differing geometries.

Catalytic activity of SsoPTP towards [^{32}P]-labeled phosphoproteins

Exogenous phosphoprotein substrates can provide a closer mimic to the presumptive endogenous substrates of SsoPTP. Therefore, a series of exogenous [^{32}P] phosphoprotein substrates were prepared and used to assay protein phosphatase activity towards phosphohydroxyl amino acids, including phosphoseryl/threonyl-casein (^{32}P-S/T-Casein), phosphotyrosyl-casein (^{32}P-Y-Casein), phosphoseryl/threonyl-myelin basic protein (^{32}P-S/T-MBP), phosphoseryl/threonyl-RCM lysozyme (^{32}P-S/T-RCML), and phosphotyrosyl-RCM lysozyme (^{32}P-Y-RCML).

The [^{32}P] phosphoprotein substrates were assayed by the procedure of McGowan and Cohen (McGowan *et al.* 1988). Each of the [^{32}P] phosphoprotein substrates mentioned above was incubated with SsoPTP at 55 °C for 10 minutes. A 120 μl volume of 20% (w/v) trichloroacetic acid (TCA) was added to the solution to precipitate proteins and centrifuged at 12, 000 x *g* for 3 minutes. A sample of the supernatant was extracted into organic solvent as a

molybdate complex and used to count the release of ^{32}P by a scintillation counter as described in Materials and Methods.

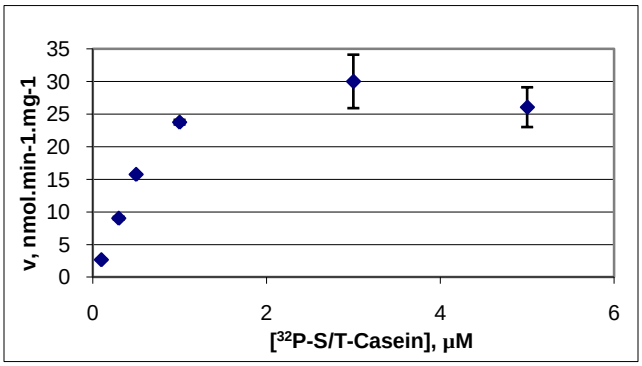
SsoPTP exhibited detectable protein phosphatase activity towards all of the exogenous [^{32}P] phosphoprotein substrates tested (Table 4-1 and Figure 4-1); thus, SsoPTP possesses dual-specific protein phosphatase activity. It was observed that SsoPTP dephosphorylated [^{32}P] phosphoprotein substrates at different rates with phosphocasein as the preferred substrate, and phosphoseryl/threonyl-RCML as the least favorable substrate (Table 4-1 and Figure 4-1). The aforementioned proteins were only used as diagnostic substrates for assessing the activity of SsoPTP against phosphoseryl/threonyl substrates versus phosphotyrosyl substrates; these exogenous substrates have no physiological relevance.

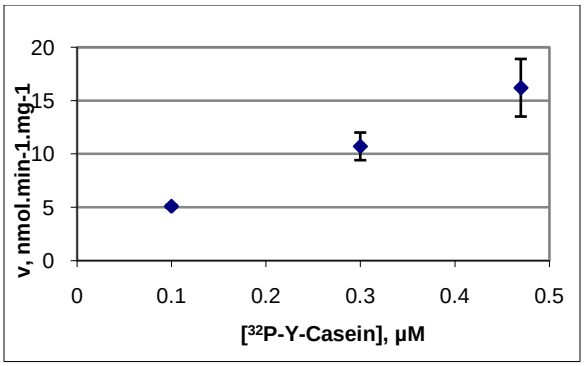
Substrate	Activity (nmol.min^{-1}.mg^{-1})							
	0.1 µM	0.3 µM	0.5 µM	1 µM	2 µM	3 µM	5 µM	8 µM
^{32}P-Y-RCML	5.11 ± 0.3	11.0 ± 0.05	11.3					
^{32}P-S/T-RCML	0.070 ± 0.001	0.194 ± 0.012	0.404 ± 0.012	0.598 ± 0.05		1.28 ± 0.04	1.49 ± 0.02	1.59 ± 0.02
^{32}P-Y-Casein	5.08 ± 0.13	10.7 ± 1.3	16.2 ± 2.7					
^{32}P-S/T-Casein	2.67 ± 0.07	9.03 ± 0.2	15.8 ± 0.05	23.8 ± 0.4		30.0 ± 4.1	26.1 ± 3.1	
^{32}P-S/T-MBP	1.74 ± 0.01	4.96 ± 0.1	6.45 ± 0.15	10.0 ± 1	9.48 ± 0.16			

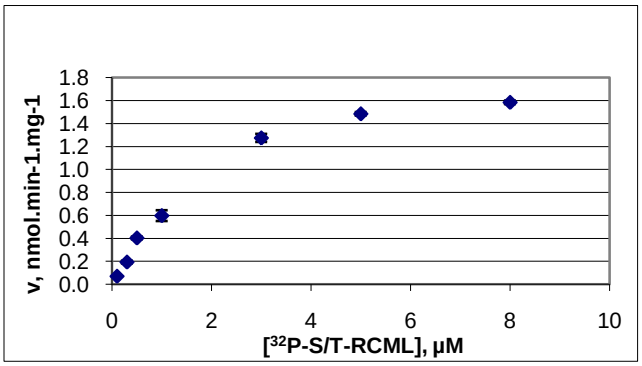
Table 4-1. Phosphatase activity of SsoPTP against exogenous [^{32}P] phosphoprotein substrates.

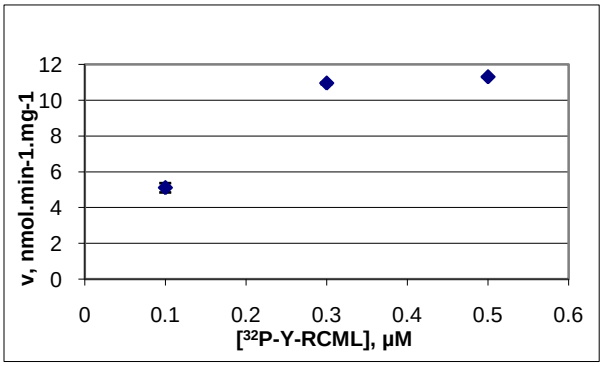
This table shows the various concentrations of ^{32}P-labeled phosphoproteins assayed with 50 ng of SsoPTP at 55°C for 10 minutes. For each concentration, the specific activity of SsoPTP was determined in nmoles of ^{32}P release per minute per mg of enzyme. The ^{32}P-labeled-serine/threonine phosphoproteins were prepared by incubating casein; reduced, carboxyamidomethylated, and maleylated lysozyme (RCML); and myelin basic protein (MBP) with [γ-^{32}P] ATP and the catalytic subunit of cAMP-dependent protein kinase (McGowan *et al.* 1988). The ^{32}P-labeled-tyrosine phosphoproteins were prepared by incubating casein and RCML with [γ-^{32}P] ATP and the *lyn* protein tyrosine kinase (Tonks *et al.* 1988) as described in Materials and Methods. The concentration of each substrate was determined by measuring the ^{32}P radioactivity present in a 5 µl aliquot of each substrate solution in CPM (counts per million)/µl and dividing the resulting amount by the specific radioactivity of the ATP used to phosphorylate the substrate (CPM/nmol). The resulting stock concentration of the ^{32}P-labeled-tyrosine phosphoproteins was much lower than that of ^{32}P-labeled-serine/threonine phosphoproteins because of the stoichiometric ratio of serine/threonine residues versus tyrosine residues on the surface of each protein. Therefore, the final concentration of ^{32}P-labeled-tyrosine phosphoproteins used in the assay was a maximum of 0.5 µM. The final concentration of each ^{32}P-labeled-serine/threonine phosphoproteins used in the assay was 2 µM, 5 µM, and 8 µM for ^{32}P-S/T-MBP, ^{32}P-S/T-Casein, and ^{32}P-S/T-RCML, respectively, as also shown in Figure 6.

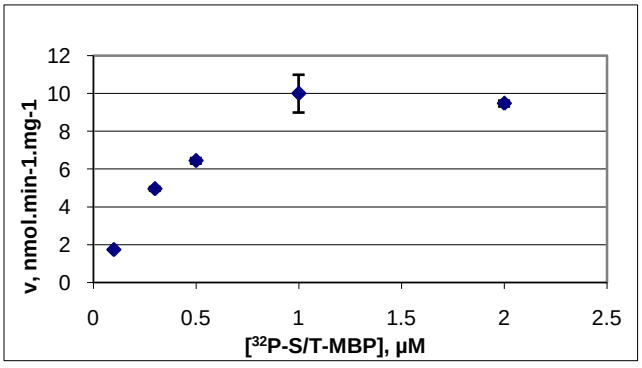
A.


v, nmol.min-1.mg-1
35
30
25
20
15
10
5
0
0
2
4
6
[32P-S/T-Casein], µM


v, nmol.min-1.mg-1
20
15
10
5
0
0
0.1
0.2
0.3
0.4
0.5
[32P-Y-Casein], µM


v, nmol.min-1.mg-1
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
[32P-S/T-RCML], µM


v, nmol.min-1.mg-1
12
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
[32P-Y-RCML], µM


v, nmol.min-1.mg-1
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
[32P-S/T-MBP], µM

B.

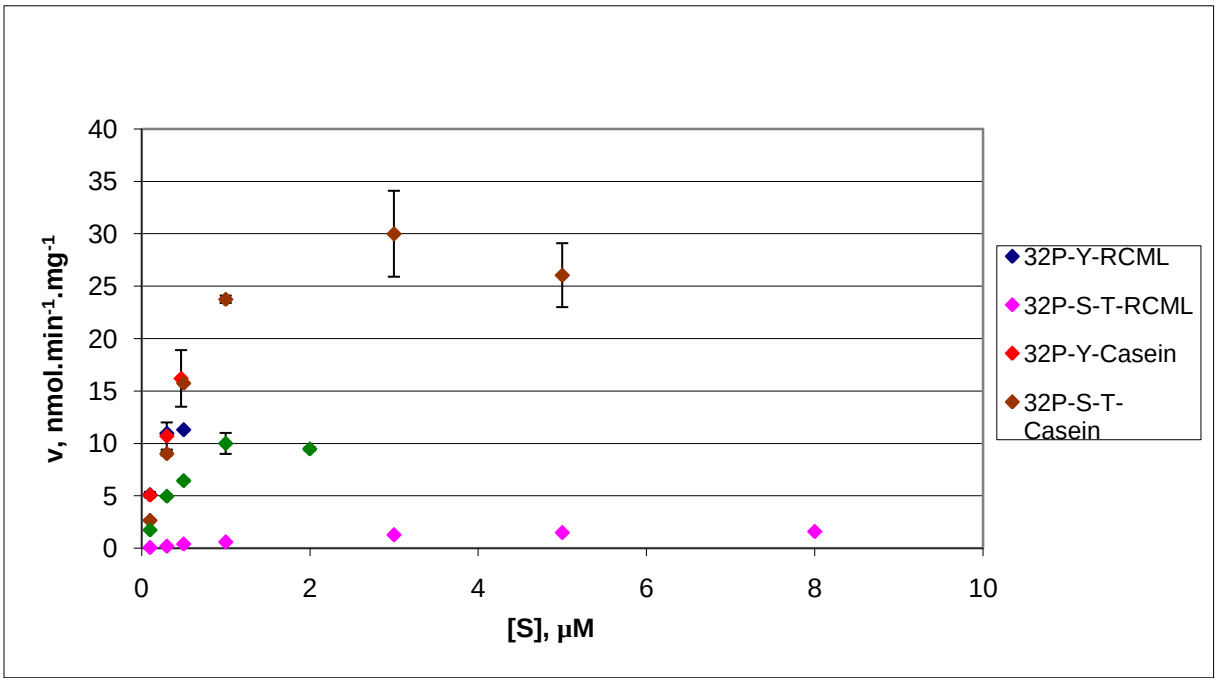


Figure 4-1. **Substrate saturation curves of SsoPTP with each [^{32}P] phosphoprotein substrates used.** The amount of SsoPTP is constant (50 ng), and the velocity, v, of the reaction in nmol.min^{-1}.mg^{-1} is determined at various substrate concentrations, [S]. (A) phosphatase activity of SsoPTP with each of the ^{32}P-labeled phosphoproteins used shown separately. (B) phosphatase activity of SsoPTP with the ^{32}P-labeled phosphoproteins used shown collectively. For details, see legend of Table 4-1.

Kinetic analysis of SsoPTP phosphatase activity

Kinetic analyses were performed with SsoPTP (Table 4-2) using *p*NPP as well as ^{32}P-labeled phosphoseryl/threonyl proteins substrates. SsoPTP displayed a 15- and 6-fold lower V_{max} toward ^{32}P-S/T-RCML, a small acidic protein, than it did toward ^{32}P-S/T-casein, another acidic protein, and ^{32}P-S/T-MBP, a small basic protein, respectively. K_m values for both ^{32}P-S/T-casein and ^{32}P-S/T-MBP were fairly comparable. The kinetic parameters k_{cat} and k_{cat}/K_m for ^{32}P-S/T-RCML were different from those of ^{32}P-S/T-Casein and ^{32}P-S/T-MBP with a 15- and 6-fold decrease in k_{cat} and a 75- and 36-fold decrease in $k_{cat}/K_{m,}$ respectively.

The kinetic parameters, K_m, k_{cat}, and k_{cat}/K_m, of SsoPTP were determined toward *p*NPP in order to compare its kinetic efficiency with several known cPTPs (Table 4-3). It was observed that the kinetic values of SsoPTP towards *p*NPP are within range of the kinetic parameters of other known cPTP members (two dual-specific phosphatases, VHR and Cdc25B, and four tyrosine-specific phosphatases, PTP1B, PTP1, LAR, and *Yersinia* PTP). The kinetic parameters of the human VHR dual-specific phosphatase and the human PTP1B were very similar to those of SsoPTP. The K_m values of the rat PTP1, rat LAR, and the bacterium *Yersinis* PTP, were similar to the K_m value of SsoPTP. No kinetic parameters of any other archaeal cPTP have been reported.

Substrate	V_{max} (nmol.min^{-1}.mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
***p*NPP**	14300	1.14	4.36	3833
^{32}P-S-T-Casein	29.6	0.0004	0.01	22500
^{32}P-S-T-RCML	2.04	0.0021	0.001	300
^{32}P-S-T-MBP	11.4	0.00032	0.003	10833

Table 4-2. **Michaelis constants for SsoPTP.** The concentration of phosphoprotein substrates were varied between 0.1 and 8 µM protein-bound ^{32}P and the concentration of *p*NPP was varied between 1 and 25 mM. Kinetic constants were extrapolated by linear regression analysis of Hanes-Woolf plots.

Enzyme	*p*NPP			Reference
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}. s^{-1}$)	
SsoPTP	1.14	4.37	3833	This study
Human VHR	1.94	5.97	3080	(Zhang *et al.* 1995)
Human Cdc25B	8.4	0.35	41.6	(Gottlin *et al.* 1996)
Human PTP1B	2.20	17.3	7900	(Sarmiento *et al.* 1998)
Rat PTP1	0.434	20.0	46000	(Denu *et al.* 1996)
Rat LAR	0.410	6.10	14000	(Pot *et al.* 1991)
Yersinia PTP	2.60	345	133000	(Zhang *et al.* 1994)

Table 4-3. Michaelis constants for different enzymes compared to SsoPTP using pNPP as a substrate. Human VHR and Cdc25B are dual-specific phosphatases; whereas human PTP1B, rat PTP1, rat LAR, and the bacterium *Yersinia* PTP are tyrosine-specific cPTPs. Prior to this study there was no report of kinetic parameters of any archaeal cPTP.

Catalytic activity of SsoPTP towards α- and β- naphthyl phosphate

The use of low molecular weight phosphomonoesters α- and β- naphthyl phosphate provides a useful and simple assay for differentiating cPTPs from dual specificity phosphatases (DSPs) *in vitro* (Howell *et al.* 1996). The phosphate group on α-naphthyl phosphate is not accessible to phosphotyrosine-specific cPTPs because the aromatic ring of α-naphthyl phosphate cannot fit in the deep and narrow active site pockets (Yuvaniyama *et al.* 1996). The β-isomer, on the other hand, has the phosphate group positioned where it can be readily accessed by phosphotyrosine-specific cPTPs as shown in Figure 4-2. Hence, phosphotyrosine-specific cPTPs hydrolyze β-naphthyl phosphate much more efficiently than α-naphthyl phosphate whereas DSPs hydrolyze both isomers with roughly equal efficiency. DSPs have active site pockets that are atypically wide and/or shallow relative to other cPTPs.

SsoPTP displayed phosphohydrolase activity toward both α- and β- naphthyl phosphate, indicating that the depth of SsoPTP active site pocket is similar to DPSs. SsoPTP hydrolyzed β-naphthyl phosphate more efficiently with a 1.5-fold increase in activity than with α- naphthyl phosphate (1.0 : 1.5 ratio of α- : β-) (Figure 4-3).

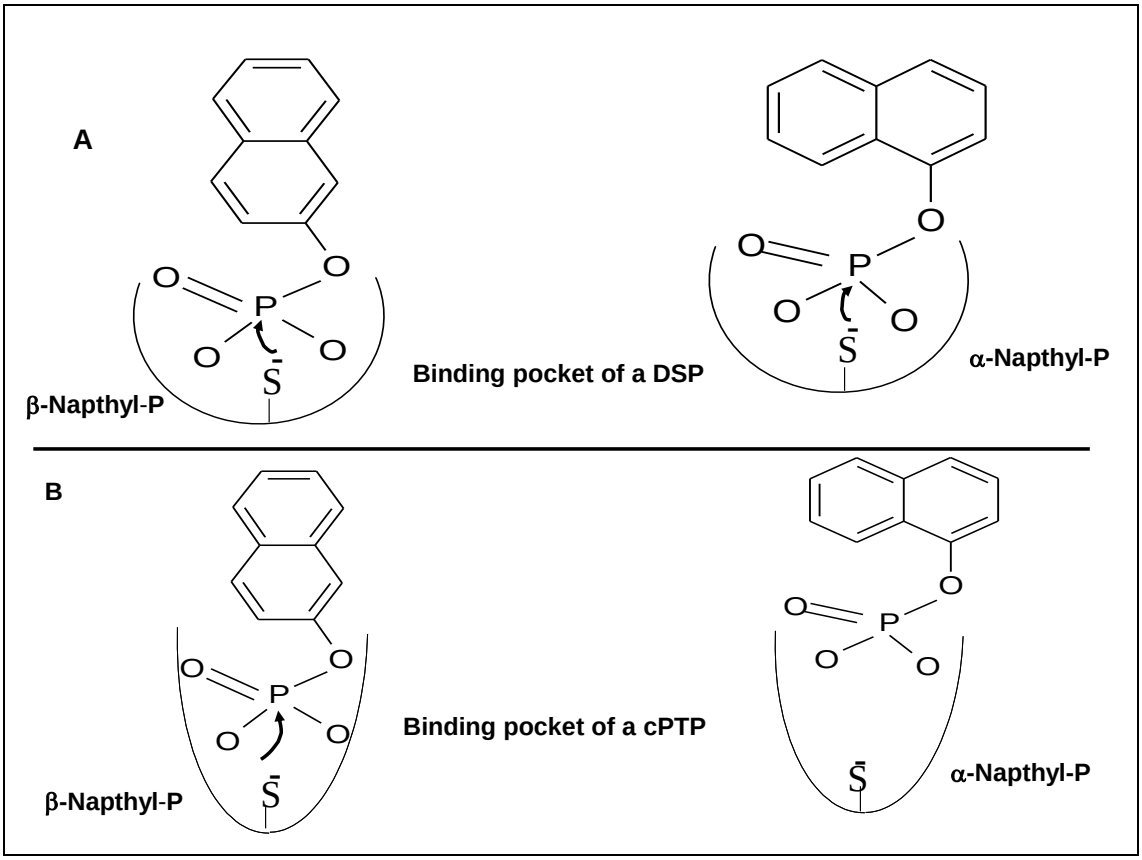


Figure 4-2. Schematic diagrams of interactions of α- and β- isomers of naphthyl phosphate with the active site pocket of DSP (A) vs. tyrosine-specific cPTP (B). The depth of an active site pocket of DSP is about 6 Å whereas the depth of an active site pocket of a tyrosine-specific cPTP is about 9 Å (Zhang 1998).

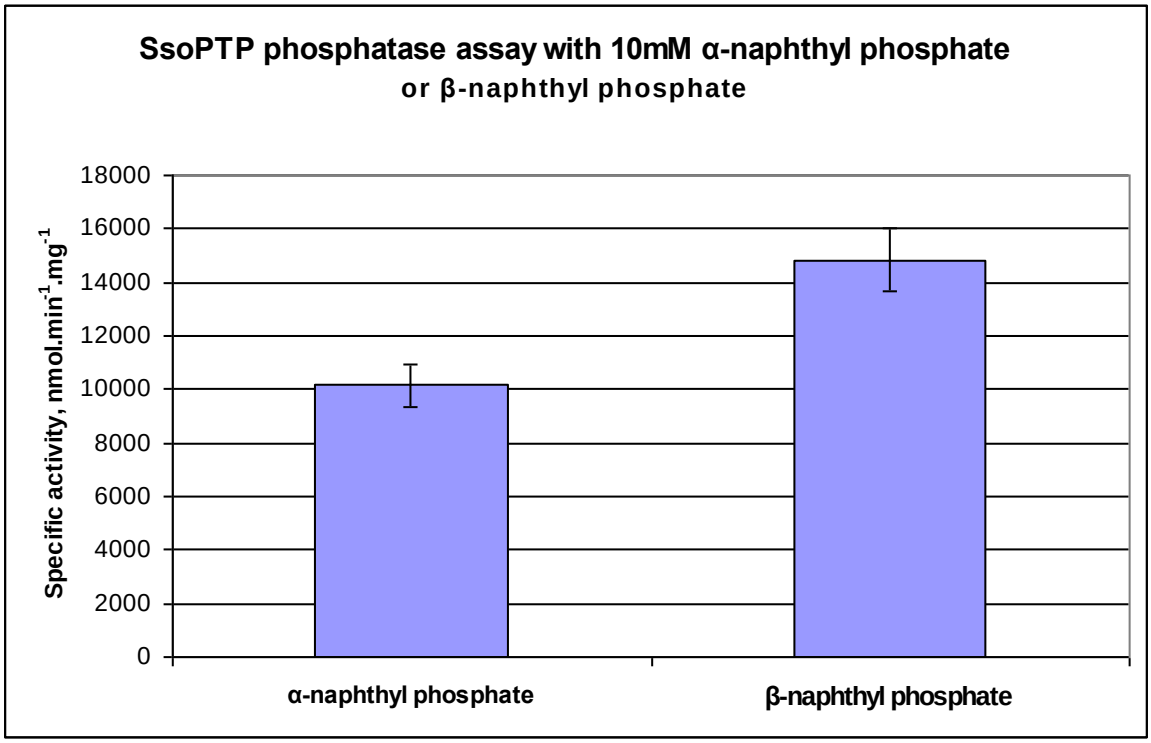


Figure 4-3. Specific activity of SsoPTP towards α- and β- naphthyl phosphate. Data was collected using Malachite green assay as described in Materials and Methods. Assays were performed in triplicates.

Previous studies performed by Kennelly and Shelton (Savle *et al.* 2000) comparing the activity of strict cPTPs and DSPs toward α- and β- naphthyl phosphate revealed that the ratio of activity of two DSPs: Cdc14 and VHR, to be 1.5 : 1.0 and 1.0 : 1.7 (α- : β-), respectively; whereas the ratio of activity of two strict cPTPs: PTP1B and Tc-PTPa, were 1.0 : 43 and 1.0 : 17.6 (α- : β-), respectively (Table 4-4). These results are consistent with the ratio of activity of SsoPTP toward α- versus β-naphthyl phosphate of 1.0 : 1.5, being a dual-specific phosphatase.

SsoPTP did not exhibit phosphohydrolase activity when tested against several phosphomonoester sugars and nucleotides including, glucose-6-phosphate, myoinositol-2-monophosphate, fructose-1,6-bisphosphate, ribose-5-phosphate, ATP, ADP, and AMP, using Malachite green assay as described for α- and β- naphthyl phosphate. The activity of SsoPTP was under the detection limit of the assay.

Enzyme	α- : β-
SsoPTP	1.0 : 1.5
Cdc14	1.5 : 1.0
VHR	1.0 : 1.7
PTP1B	1.0 : 43
Tc-PTPa	1.0 : 17.6

Table 4-4. Ratio of activity of SsoPTP toward α- and β-naphthyl phosphate compared with two DSPs: Cdc14 and VHR; and two tyrosine-specific cPTPs (strict PTPs): PTP1B and Tc-PTPs. The catalytic activity of SsoPTP is similar to VHR and Cdc14 in dephosphorylating both isomers of naphthyl phosphate with roughly equal efficiency. α- : β- are the ratio of activity of cPTPs toward α-napthyl phosphate versus β-naphthyl phosphate. Data for Cdc14, VHR, PTP1B, and Tc-PTPa are taken from Savle *et al.* (2000).

CHAPTER V

Determination of the chemical mechanism of SsoPTP

Protein tyrosine phosphatases employ a cysteine residue in the active site signature motif to catalyze the formation of a thiophosphate-enzyme intermediate as part of the two-step mechanism established for all members of the cPTP family. Previous studies showed that altering the active site Cys using an alkylating agent such as iodoacetate, which selectively reacts with thiol groups, causes a complete loss in catalytic activity of cPTPs (Guan *et al.* 1991; Pot *et al.* 1991; Zhou *et al.* 1994). The invariant Arg residue in cPTP signature motif HCxxGxxR is involved in substrate recognition and stabilization of the transition state. An essential Asp residue on the surface loop is involved in protonating the leaving group thereby acting as a general acid catalyst. In the second step, this same Asp acts as a general base by making a water molecule more nucleophilic to carry out the hydrolysis of the phosphoenzyme intermediate and the release of free enzyme and inorganic phosphate. Altering the conserved Asp residue or the Arg residue was shown to abolish the catalytic activity of cPTPs (Zhang *et al.* 1994; Zhou *et al.* 1994; Kennelly 1999; Liang *et al.* 2007). The conserved active site His residue was shown to be involved in stabilizing the active site Cys thiolate anion (Zhang *et al.* 1993; Zhang *et al.* 1994). An invariant Gln residue, which plays a role in positioning a water molecule for the direct attack and hydrolysis of the phosphoenzyme intermediate, was shown to dramatically decrease the activity of the human PTP1B and the bacterium *Yersinia* PTP (Zhao *et al.* 1998; Xie *et al.* 2002; Peters *et al.* 2004).

To determine whether the chemical mechanism of SsoPTP parallels that of known members of the cPTP family, the following residues were selectively altered: Asp^{69}, His^{95},

Cys^{96}, Arg^{102}, Arg^{130}, Gln^{135}, and Gln^{139} to verify whether each residue is essential for catalysis (Figure 5-1).

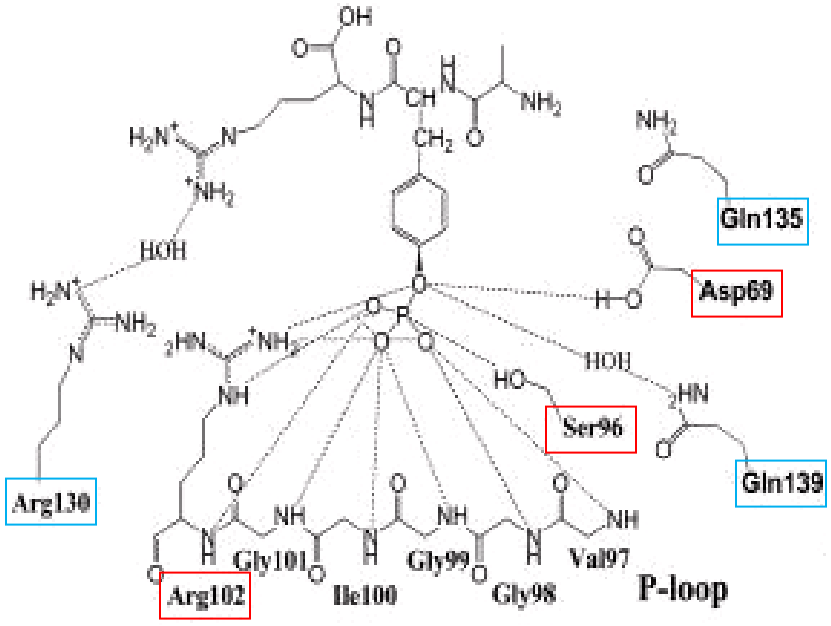


Figure 5-1. Schematic diagram of C96S-SsoPTP active site interactions with A(p)YR phosphopeptide. The invariant residues, H^{95}, C^{96}, R^{102}, and D^{69}, in cPTP signature motif, $DX_{27}HCX_5R$, were chosen for mutagenic alteration, and are shown in red boxes (except for H residue which is not shown in this diagram, and C residue is replaced with S residue). The invariant residues, R^{130}, Q^{135}, and Q^{139}, are shown in blue boxes, and were also chosen for mutagenic alteration based on their interaction with the phosphosubstrate depicted in this diagram. This diagram was adapted from Chu et al. (2007).

Construction of SsoPTP protein variants by site-directed mutagenesis

Site-directed mutagenesis was employed to construct the SsoPTP protein variants, C96S, D69A, R102K(A), H95A, Q139A(E,D,S,N), R130K(A), and Q135A, as described in Materials and Methods. SsoPTP protein variants were purified using IMAC as described for the wildtype (WT) enzyme. Phosphohydrolase activity of each SsoPTP protein variant was determined using *p*NPP as substrate.

Enzymatic analysis of SsoPTP protein variants

Mutagenic alteration of the active site Cys^{96} and Arg^{102} residues abolished catalytic activity as predicted (Table 5-1 and Figure 5-2) (Zhang *et al.* 1994; Zhang *et al.* 1994; Zhou *et al.* 1994; Jeon *et al.* 2002). The Asp^{69} and His^{95} mutations to Ala residue resulted in a dramatic decrease in activity as was previously shown (Zhang *et al.* 1993; Zhang *et al.* 1994; Zhang *et al.* 1994). Substituting the invariant Gln^{139} residue to Ala residue produced a catalytically impaired enzyme (Table 5-1 and Figure 5-2). Since SsoPTP-Q139A caused a dramatic decrease in catalytic activity, attempts to restore the catalytic activity of SsoPTP by substituting Gln^{139} with a residue that possess similar size and hydrogen bonding capability such as Glu, Asp, Ser, or Asn were tested. However, those attempts proved unsuccessful in restoring the catalytic activity of Gln^{139}-altered SsoPTP. Altering the invariant Arg^{130} to Ala residue resulted in a variant with 30% less activity compared to the Lys substitution, while the latter and the invariant Gln^{135} residue showed a little effect on catalytic activity.

Enzyme form	% Activity
	*p*NPP
WT	100
C96S	0.0052
R102K	0.031
R102A	0.15
D69A	2.5
H95A	7.7
Q139A	5.5
Q139E	4.4
Q139D	2.6
Q139S	3.6
Q139N	3.9
R130A	37.9
R130K	72.7
Q135A	84.7

Table 5-1. **Effect of mutagenically produced alterations on the catalytic efficiency of SsoPTP.** The modified proteins were assayed for phosphatase activity toward 10 mM *p*NPP. Altering the invariant residues, C^{96}, R^{102}, D^{69}, H^{95} abolished the catalytic activity of SsoPTP as predicted for the members of the cPTP family. Altering the invariant residue Q^{139} abolished SsoPTP activity, while changing R^{130} and Q^{135} showed little effect on the activity of SsoPTP.

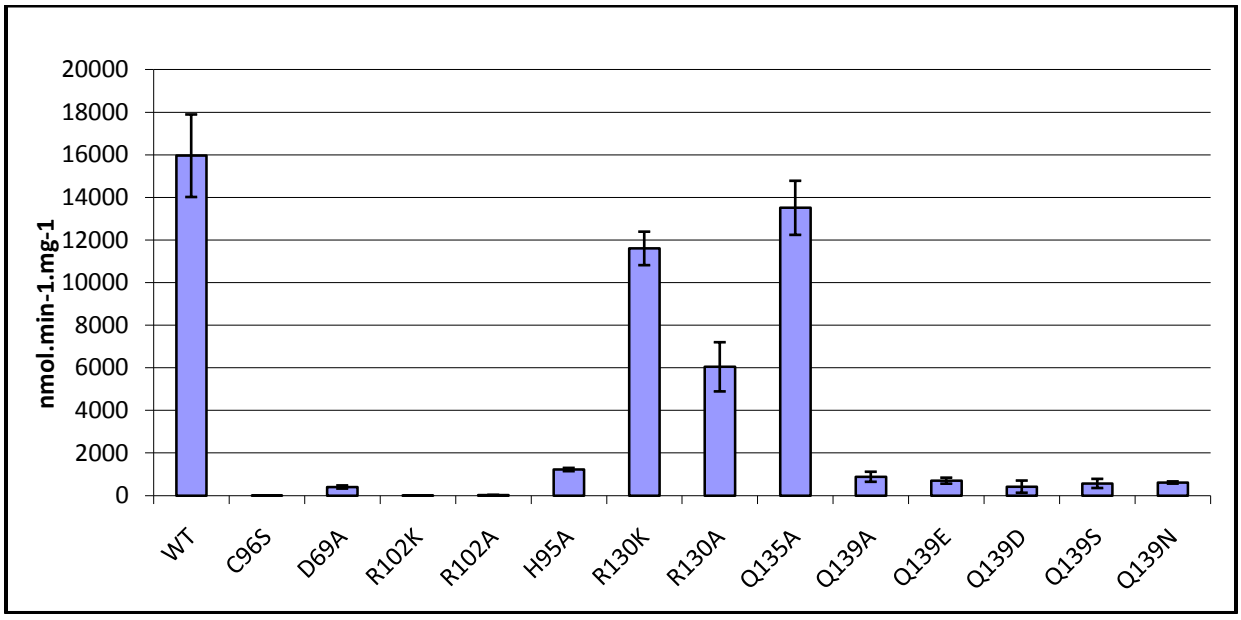


Figure 5-2. Effect of mutagenically produced alterations on the specific activity of SsoPTP using *p*NPP as a substrate. Each SsoPTP protein variant was tested with 10 mM *p*NPP under similar condition previously mentioned for the WT enzyme. For details, see legend for Table 5-1.

Amino acid sequence alignment of SsoPTP and other members of the PTP family from archaea, bacteria, yeast, and mammalian organisms showed that the Gln residues (equivalent to Gln^{139} in SsoPTP) are conserved in archaea, whereas the second Gln residue (Gln^{135} in SsoPTP) is replaced by Glu, a conservative substitution. However, altering the Gln^{139} to Glu did not restore the catalytic activity of SsoPTP as mentioned above. In human tyrosine-specific cPTPs, Gln residues (Gln^{135} in SsoPTP) are conserved compared to the other Gln residues (Gln^{139} in SsoPTP) (Figure 5-3). In human dual-specific cPTPs, Gln residues (Gln^{139} in SsoPTP) are conserved whereas Gln residues (Gln^{135} in SsoPTP) are replaced by Gly or Glu or Asn.

Mutagenic analyses of Gln^{262} residue in the human PTP1B (Xie *et al.* 2002; Peters *et al.* 2004), and Gln^{446} in the bacterium *Yersinia* cPTP (Zhao *et al.* 1998) (equivalent to Gln^{135} in SsoPTP) showed a dramatic decrease in catalytic activity similar to the results obtained from alerting Gln^{139} and not Gln^{135}. Active site three-dimensional alignment of crystal structure of SsoPTP with crystal structures of PTP1B and *Yersinia* cPTP revealed that the Gln^{135} and Gln^{139} of SsoPTP are aligned with Gln^{262} and Gln^{266} of PTP1B, and Gln^{446} and Gln^{450} of *Yersinia* cPTP, respectively (Figure 5-4), which is consistent with their primary amino acid alignment (Figure 5-3). This may suggest altered roles of the two invariant glutamine residues among organisms. No additional studies on the two glutamines have been reported other than those aforementioned.

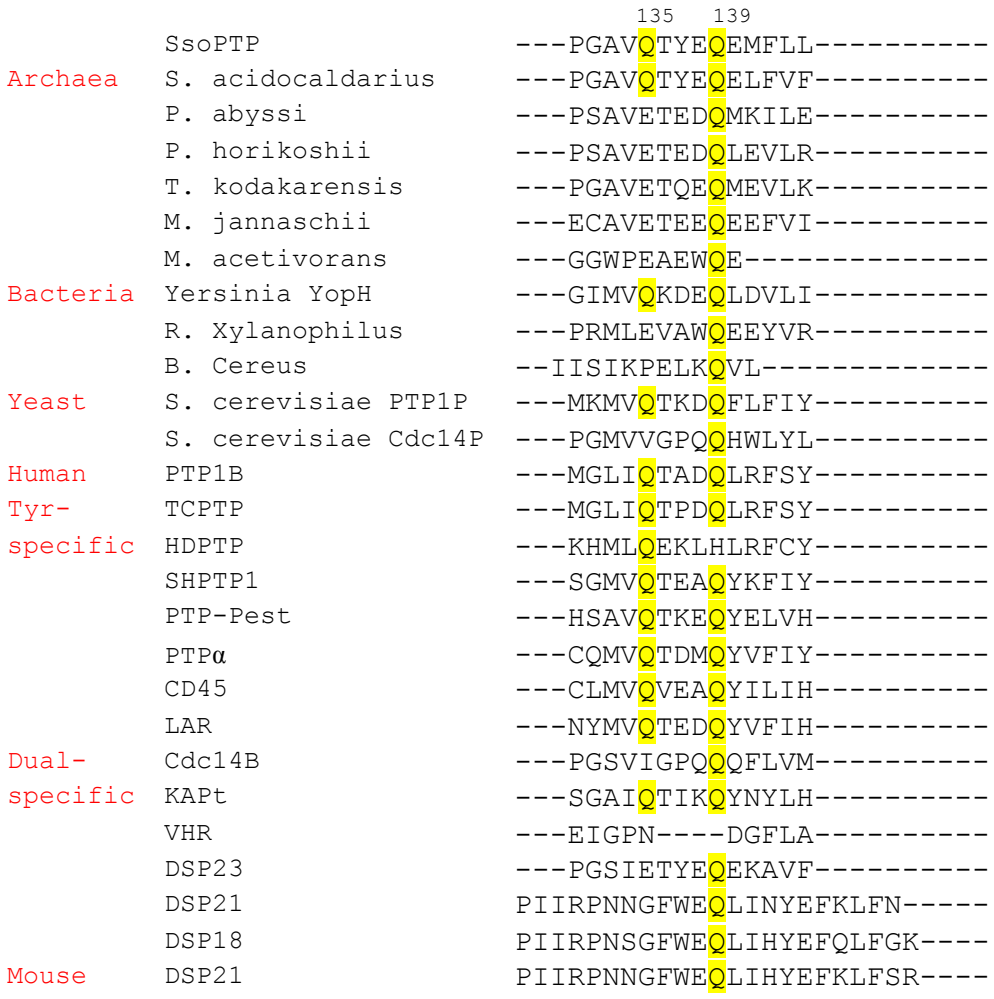
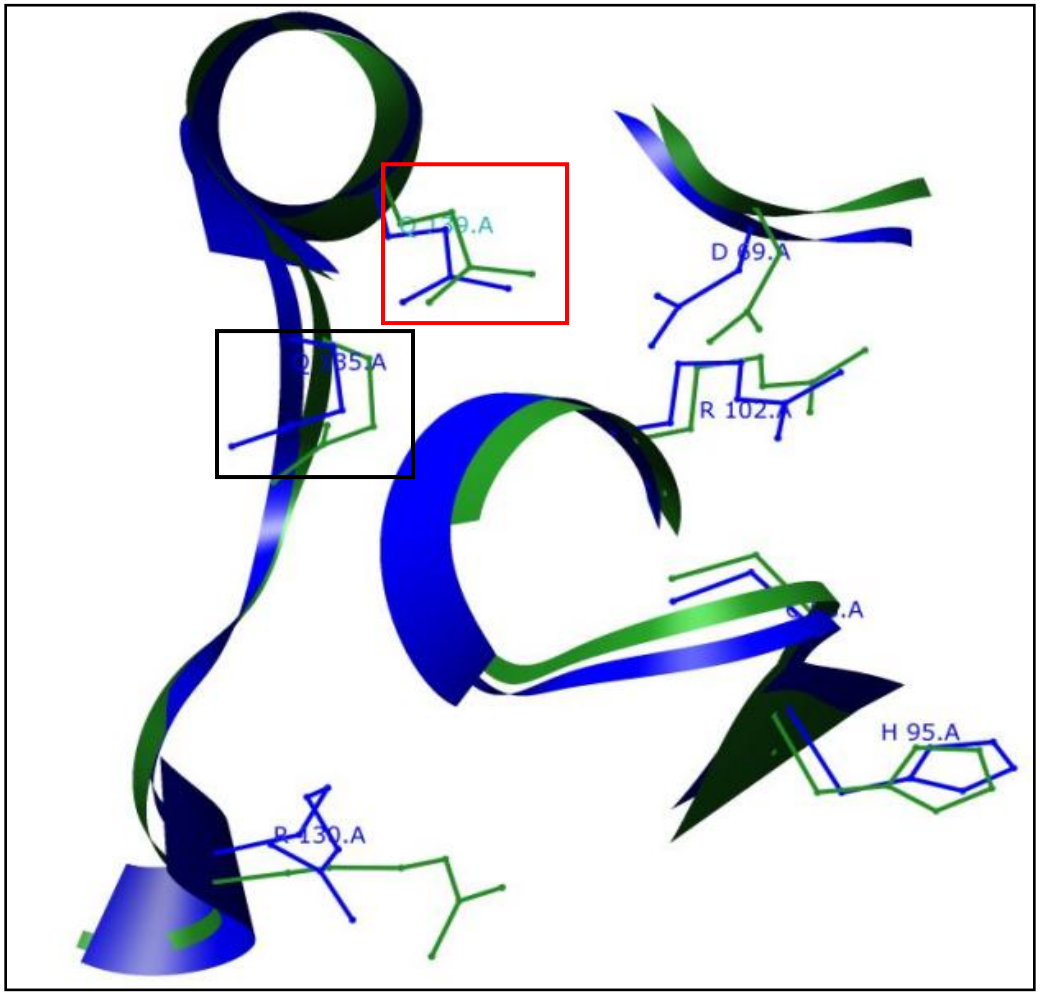


Figure 5-3. Primary amino acid sequence alignment of 6 archaeal PTPs, 3 bacterial PTPs, 2 yeast PTPs, 14 human PTPs, and 1 mouse PTP with SsoPTP. The two conserved glutamine residues are highlighted in yellow.

A.



B.

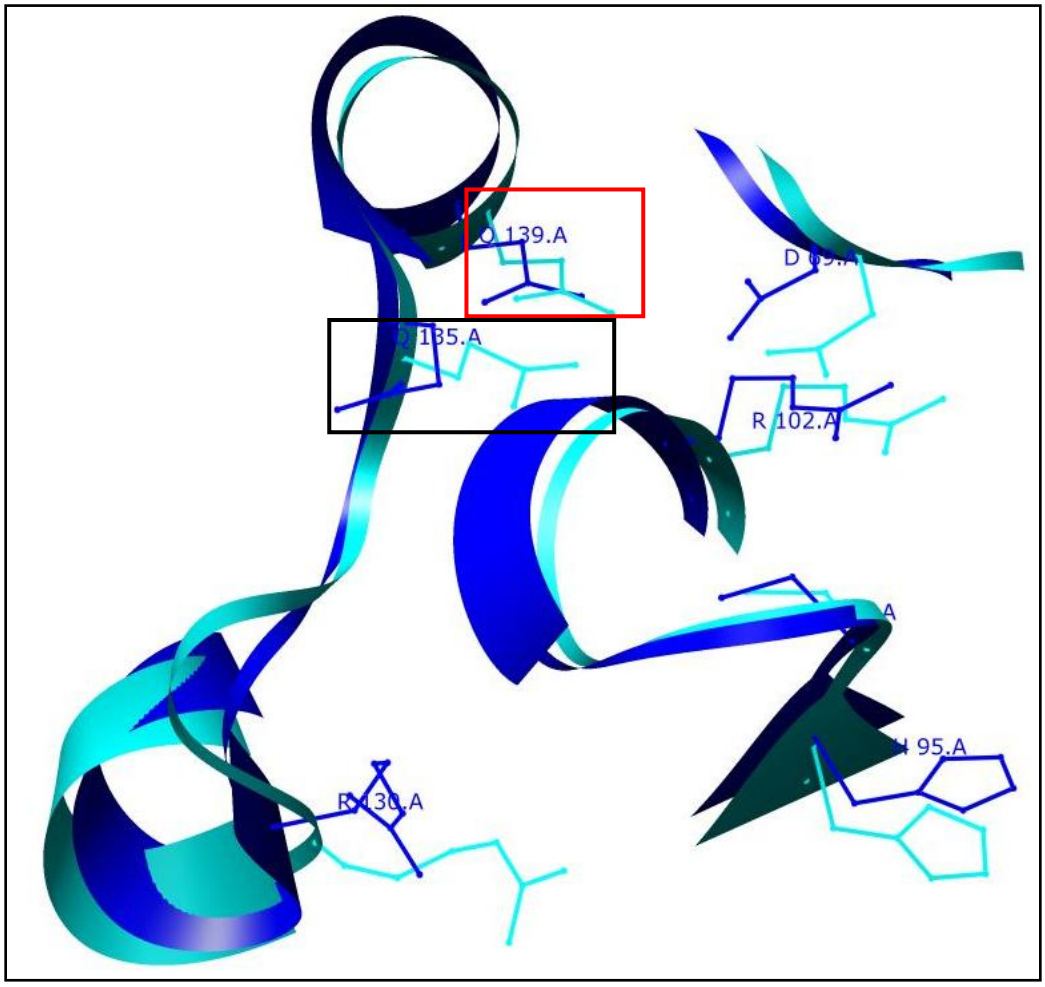


Figure 5-4. Active site three-dimensional structure alignment of SsoPTP, represented in blue ribbon with: (A) PTP1B, (B) *Yersinia* cPTP. Catalytic residues are shown as ball and stick representation with numbering corresponding to SsoPTP: R^{130}, R^{102}, Q^{135}, Q^{139}, C^{96}, H^{95}, and D^{69}. (A) Gln^{139} of SsoPTP and Gln^{266} of PTP1B are boxed in red, and Gln^{135} of SsoPTP and Gln^{262} of PTP1B are boxed in black. (B) Gln^{139} of SsoPTP and Gln^{450} of *Yersinia* cPTP are boxed in red, and Gln^{135} of SsoPTP and Gln^{466} of *Yersinia* cPTP are boxed in black. Images were generated using CHIMERA.

Chemical rescue of catalytic activity of SsoPTP protein variants

A possibility for loss of enzymatic activity of SsoPTP protein variants is altering an essential residue for catalysis. To determine if this is the case, a chemical rescue approach was used. The side chain of amino acid residues is involved in catalysis. Altering a residue or replacing a reactive side chain with a non-reactive one can abolish catalysis. Adding the reactive side chain in the reaction buffer, in a form of a small molecule (rescue agent), can sometimes rescue catalysis by providing the essential reactive groups. To rescue the activity of SsoPTP-H95A, SsoPTP- R102A(K) or R130A(K), SsoPTP- C96S, SsoPTP- D69A, and SsoPTP-Q135A or SsoPTP-Q139A, buffers containing one of the following compounds was used: imidazole, guanidine HCl, β-mercaptoethanol, acetic acid or bicarbonate, and acetamide, respectively.

SsoPTP wild type and SsoPTP protein variants were assayed against *p*NPP and rescue agents at different concentrations were added (Table 5-2). As can be seen in Figure 5-5A, increasing amounts of exogenous guanidine hydrochloride enhances the activity of SsoPTP-R102A, but not R102K, when compared to their activities without guanidine hydrochloride. Guanidine concentration of 100 mM caused a 2.4-fold increase in activity of SsoPTP-R102A (Figure 5-5A). There was little to no effect on the activity of the wild type enzyme at or below 100 mM guanidine. At higher guanidine concentrations, a decrease in the activity of the wild type enzyme was observed (Figure 5-5B).

The invariant Arg^{102} residue was shown to play a role in stabilizing the transition state by forming salt bridges with the phosphate group on the substrate. Substituting Arg^{102} with Ala residue replaces the charged guanidino group side chain of Arg with unpolar and uncharged –CH_2 side chain of Ala; thus, R^{102} loses its ability to interact with the substrate. Adding exogenous

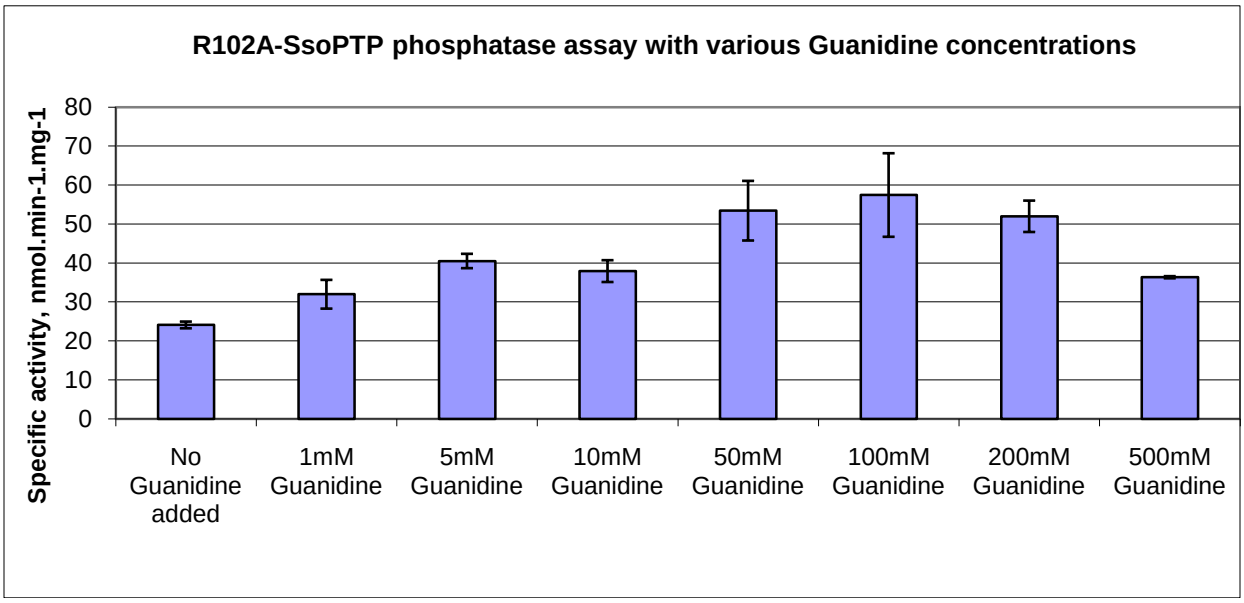
charged guanidine into the buffer (pK_a of guanidine is 13.6, therefore it is positively charged at pH 6) can mimic the guanidino group in the active site cavity and enhance the activity of the enzyme. However, the activity of SsoPTP-R102A in guanidine was minimal when compared with the activity of the WT enzyme. This might be explained by the freedom of motion of guanidine in the active site compared to the covalently attached side chain of arginine with the proper orientation for interaction with the substrate.

Exogenous guanidine was unable to enhance the activity of the SsoPTP-R102K possibly because of the long charged side chain of the lysine residue occupying the active site, and thus, minimizing the interaction of free guanidine with the substrate in the active site.

All other rescue agents listed in Table 5-2 were unable to enhance or increase the activity of the rest of the SsoPTP protein variants.

Chemical rescue approach was employed in several studies including, *E. coli* Ornithine Transcarbamylase, where the rate of a mutant form of OTCase-R57G was enhanced 2000-fold by exogenous guanidine (Rynkiewicz *et al.* 1996); rat pancreatic carboxypeptidase-R127A was partially rescued by guanidine derivatives (Phillips *et al.* 1992); the *Pseudomonas putida* (S)-Mandelate dehydrogenase-H274G was partially restored by the addition of exogenous imidazoles to ~0.1% of the activity of the WT (Lehoux *et al.* 1999); rat trypsin-D189S was partially rescued by exogenous acetate as much as 300-fold (Perona *et al.* 1994). No chemical rescue studies on protein phosphatases have been reported.

A.



B.

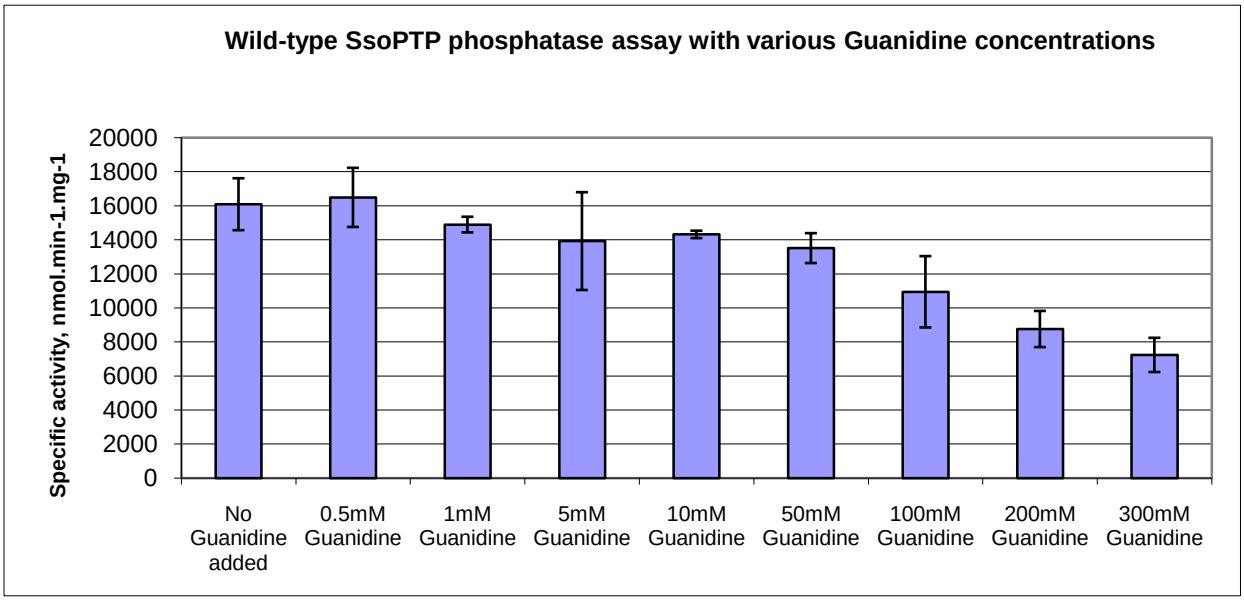


Figure 5-5. Effect of Guanidine on the activity of SsoPTP-R102A (A) and SsoPTP WT (B). The activity of SsoPTP-R102A and SsoPTP WT was measured using 10mM *p*NPP as a substrate. Assays were performed in three sets of duplicates.

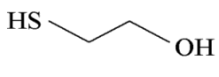
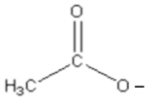
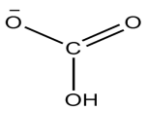
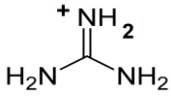
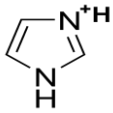
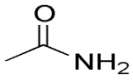
Enzyme form	Chemical added	[Chemical], mM	Fold increase of activity
C96S	β-mercaptoethanol  pka 9.5	1, 10, 100, 300, 500, 1000	ND
D69A	Acetic acid  pka 4.76	1, 100, 300, 500, 1000, 2000, 3000	ND
	Bicarbonate  pka 10.24	100, 500	ND
R102A R102K R130A R130K	Guanidine-HCl  pka 13.6	0.5, 1, 5, 10, 50, 100, 200, 300, 500	2.4 ND ND ND
H95A	Imidazole  pka 6.99	1, 5, 10, 50, 100, 200	ND
Q139A Q135A	Acetamide  pka 17	10, 50, 100, 200, 500	ND ND

Table 5-2. Chemical rescue approach to restore the activity of SsoPTP protein variants.
The structure of each chemical added to the buffer is drawn to illustrate the structural similarities with the side chain of the residue that has been altered. The pka of each chemical is also shown to predict its charge at pH 6 of the buffer used in SsoPTP assay. The concentration of each chemical in mM used in the assay is shown. ND stands for not detected.

Partial proteolysis of SsoPTP and its protein variants

Another possible explanation for loss of enzymatic activity of SsoPTP protein variants is protein misfolding. To check whether altering the residues mentioned above caused a conformational change, partial proteolysis was employed. A misfolded protein is more likely to be accessible to a protease and thus more rapidly degraded, and potentially at different sites, than a properly folded protein. Therefore, the rate and pattern by which a protein is hydrolyzed can be compared to the rate of its protein variants to give information about the folding of the protein variant with respect to its native form. Trypsin, which is a serine protease that specifically cleaves at the carboxylic side chain of lysine and arginine, was chosen to hydrolyze SsoPTP and its protein variants.

Each of the wild type enzyme and protein variants was incubated with trypsin at 37°C for 0, 15, 30, and 60 minutes. An aliquot of the digestion mixture was immediately heated to 100°C for 5 minutes upon the addition of 1x SDS loading buffer to stop the reaction. Each reaction aliquot was subjected to SDS-PAGE gel as described in Materials and Method. The resulting digestion pattern of the wild type enzyme was similar to the digestion pattern of each of the protein variants, which indicates similar rate of hydrolysis, and thus similar fold (Figure 5-6).

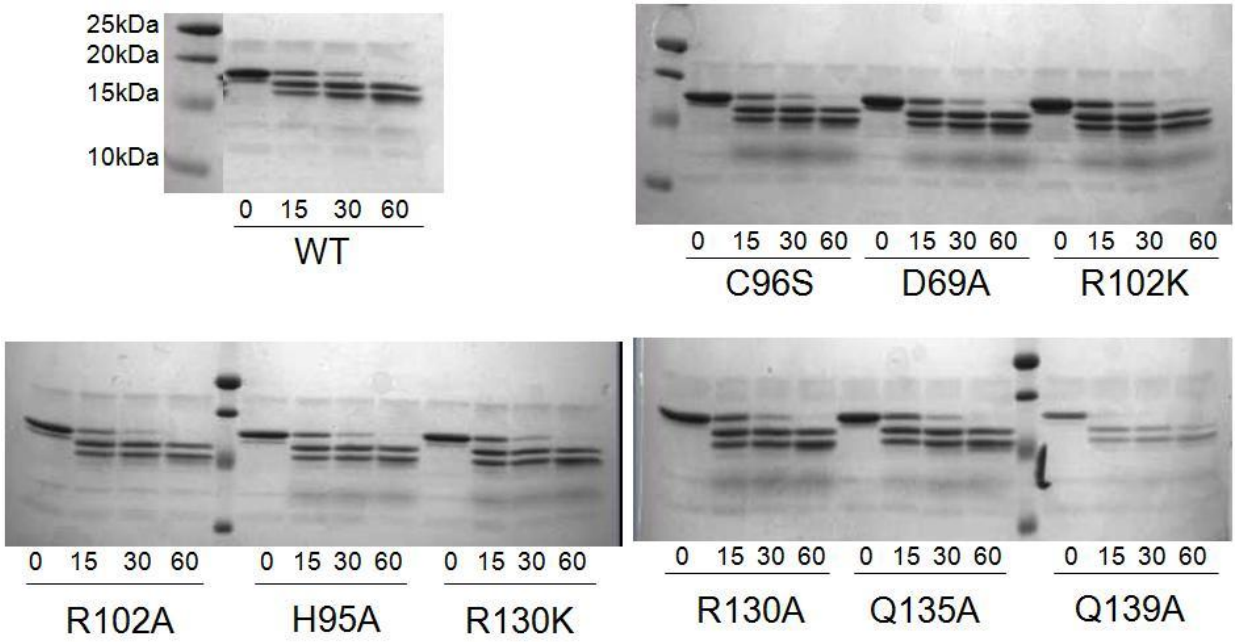


Figure 5-6. Comparison of partial tryptic digest of SsoPTP WT with each of its protein variants. Shown above are coomassie blue staining of 15% SDS-PAGE gels of aliquots of 2.4 µg of WT enzyme and SsoPTP protein variants, each digested with 0.64 µg trypsin taken from the reaction mixture performed at 37˚C for 0, 15, 30, and 60 minutes as described in Materials and Methods. The first gel on the upper left is SsoPTP WT digested at 0, 15, 30, and 60 minutes as indicated on the gel. The gel on the upper right has three digested SsoPTP protein variants: SsoPTP-C96S, SsoPTP-D69A, and SsoPTP-R102K, respectively. The two gels on the bottom are as follows (from left to right): SsoPTP-R102A, SsoPTP-H95A, SsoPTP-R130K, SsoPTP-R130A, SsoPTP-Q135A, and SsoPTP-Q139A, respectively. Bands of digested SsoPTP-Q139A, are less intense because its concentration is less than the concentration of all other proteins in the assay. Molecular weight protein standard are labeled on the far left lane of the WT gel. Each of the other gels has the same molecular weight standards as labeled for the WT lane.

CHAPTER IV

Isolation of endogenous SsoPTP substrates by substrate trapping and identification by mass spectroscopy

Previous studies have shown the presence of tyrosine-phosphorylated polypeptides in *S. solfataricus* by employing phosphoamino acid-directed antibodies (Smith *et al.* 1997; Kennelly 2003); however, the identities of the putative phosphotyrosyl proteins were not determined. One potential approach for the direct identification of possible SsoPTP substrates is substrate trapping. This method relies on the ability of a mutagenically altered or catalytically impaired enzyme to bind substrates in a stable, dead-end complex, enabling capture of the PTP-substrate complex (Jeon *et al.* 2002; Xie *et al.* 2002; Agazie *et al.* 2003; Liang *et al.* 2007).

Based on the known catalytic mechanism of cPTPs, mutational alteration of the active site Cys residue to Ser or the conserved general acid Asp residue to Ala or both can form a possible substrate-trapping protein variant (Liang *et al.* 2007). Replacing the active site Cys residue with Ser can render the enzyme completely inactive, whereas altering the general acid Asp residue to Ala can reduce the rate of hydrolysis of phosphopeptides by several orders of magnitude (Milarski *et al.* 1993; Sun *et al.* 1993; Flint *et al.* 1997; Liang *et al.* 2007). In general, mutational alteration of the general acid Asp to Ala in other PTPs resulted in higher affinity toward phosphorylated protein substrates than the Cys to Ser substitution (Flint *et al.* 1997; Xie *et al.* 2002; Liang *et al.* 2007). Since the general acid Asp to Ala mutant can still possess residual phosphatase activity, altering the nucleophilic Cys to Ser can render the double protein variant inactive, which would produce an improved substrate-trapping mutant (Agazie *et al.* 2003; Wu *et al.* 2006).

Previous studies on PTP1B have used substrate trapping protein variants C215S and D181A to successfully bind to a PTP1B substrate, the EGF receptor (Milarski *et al.* 1993). The double protein variant D195A/C227S of PTPN22 was used to identify several potential substrates in lysates from pervanadate-stimulated Jurkat cells (Wu *et al.* 2006). The inactive CD45 C828S protein variant bound tightly to CD3 zeta chain, which then was identified as its substrate (Furukawa *et al.* 1994). In the *Archaea, Tk*-PTP-C93S protein variant was reported to bind to three proteins from *Thermococcus kodakaraensis* KOD1 cell lysates *in vitro*. Those proteins were identified as the cellular substrates of *Tk*-PTP: rtcB, a member of RNA terminal phosphate cyclase operon, phenylalanyl-tRNA synthetase β-chain (FTS), and phosphomannomutase (PMM) (Jeon *et al.* 2002).

A substrate trapping approach was employed to identify possible substrates for SsoPTP endogenous to *S. solfataricus*.

Cultures of *Sulfolobus solfataricus*

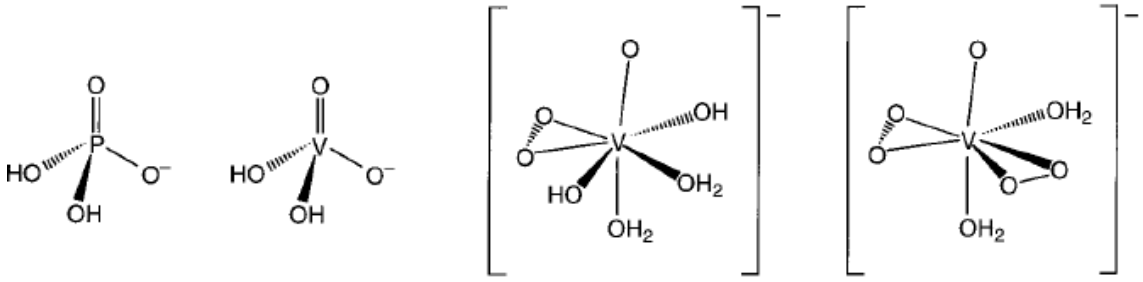
Cells of *S. solfataricus* were cultured aerobically at 72°C in 16 x 250 ml media (4 L total) for 3 - 5 days until the OD_{540} was within 0.9 - 1.0 range. Before harvesting the cells, 1 mM pervanadate was added for 30 minutes to cell cultures. Pervanadate, which was freshly prepared by mixing equal amounts of 200 mM sodium orthovanadate and hydrogen peroxide, is an irreversible PTP inhibitor, which oxidizes the sulfur atom of the active site Cys to form a sulfonic acid. However, it is unstable and it can break down into vanadate and peroxides. Adding a reducing agent such as dithiothreitol (DTT) can also reduce pervanadate to vanadate (Figure 6-1). Vanadate, on the other hand, is a reversible PTP inhibitor that reacts with the active site Cys to form cystiene-vanadate complex similar to phosphocystiene enzyme intermediate (vanadate is

a phosphate analog) (Figure 6-1) (Huyer *et al.* 1997). Collectively, adding pervanadate (vanadium peroxide complex) was shown to increase the level of tyrosine phosphorylation caused by the inhibition of the endogenous PTPs (Kadota *et al.* 1987; Fantus *et al.* 1989; Heffetz *et al.* 1990; Zick *et al.* 1990) or oxidative stress caused by peroxides.

A recent study by Maaty *et al.* was conducted on the effect of oxidative stress on *S. solfataricus* caused by hydrogen peroxide. Microarray analysis revealed that about 102 mRNA transcripts were regulated by 1.5-fold after exposure to 30 mM H_2O_2 for 30 minutes (Maaty *et al.* 2009). In addition, CyDye based 2D-DIGE analysis showed an increase in protein abundance and posttranslational modification including phosphorylation after 30 and 105 minutes H_2O_2 exposure.

Therefore, adding vanadium peroxide to growing cultures of *S. solfataricus* for 30 minutes can increase the level of endogenous protein phosphorylation; and thus, a higher chance of capturing a potential substrate for SsoPTP by substrate trapping can be achieved.

A.



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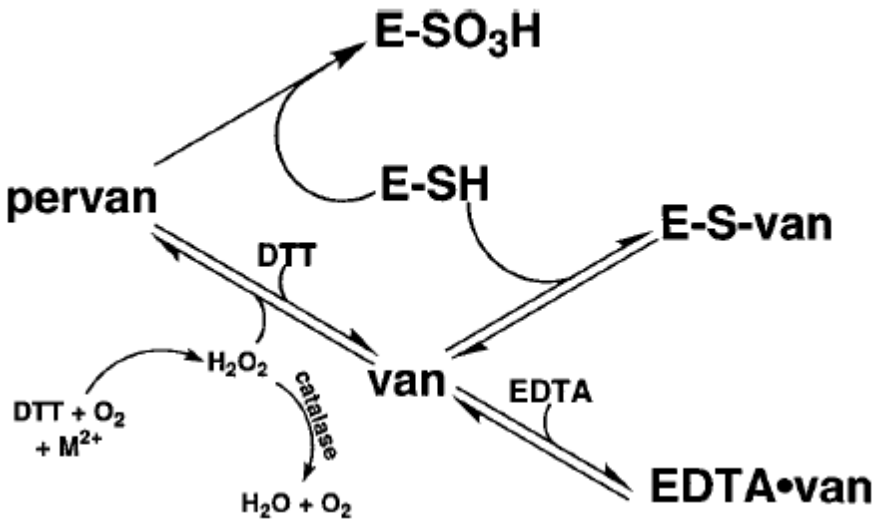


[fair use]

Figure 6-1. Protein tyrosine phosphatase inhibition by vanadate and pervanadate. (A) The structure of phosphate, vanadate, and pervanadate are shown. Vanadate is an analog of phosphate (tetrahedral structure), and thus it acts as a competitive inhibitor of PTPs by binding to active site cysteine residue. Pervanadate irreversibly oxidizes cysteine residue to form sulfonic acid, and inactive the enzyme. (B) The mechanism of inhibition of PTPs by vanadate and pervanadate is depicted in this diagram. Pervandate (pervan), which is a vanadium peroxide complex, is formed by oxidizing vanadate (vanadate and hydrogen peroxide (H_2O_2)). Pervanadate oxidizes the reduced active site cysteine (E-SH) to form sulfonic acid (E-SO_3H). Dithiothreitol (DTT) reduces pervandate to vanadate (van), and the latter is chelated by EDTA to form EDTA.van complex. Vanadate interacts reversibly with the active site cysteine residue to form cysteine-vanadate complex (E-S-Van) (Huyer *et al.* 1997).

Preparation of *S. solfataricus* whole cell lysates

Whole cell lysates of *S. solfataricus* were prepared as described in Materials and Methods. The suspension buffer contained both 5 mM iodoacetic acid and 1 mM EDTA, to irreversibly inactivate any remaining PTPs that are still active after pervanadate treatment, and chelate the remaining vanadate, respectively. Cell lysates were then treated with 10 mM DTT to inactivate any unreacted iodoacetic acid and pervanadate.

Construction of substrate trapping protein variant of SsoPTP

A substrate trapping protein variant, SsoPTP-C96S/D69A, was constructed to isolate possible SsoPTP substrates present in the lysates of *S. solfataricus* by site-directed mutagenesis as described in Materials and Methods. The protein was purified in the same manner as described for the WT enzyme.

Binding of SsoPTP and its protein variants to NHS-activated Sepharose 4 Fast Flow

The wild type enzyme and SsoPTP-C96S/D69A were coupled to NHS (N-hydroxysuccinimide)-activated Sepharose beads as described in Materials and Methods. This resin can form amide linkages between the $–NH_2$ group on proteins and N-hydroxysuccinimide. Therefore, attached proteins to the resin can resist high salt and pH changes, and remain coupled to the beads. A negative control of beads alone (with no protein ligands added) was prepared with the same manner as beads-bound WT and SsoPTP-C96S/D69A. This control served as a determinant of non-specific interaction of *S. solfataricus* proteins that were present in the resin alone column.

Elution of adherent *S. solfataricus* proteins

Equal amounts of *S. solfataricus* cell lysates, pretreated with 1 mM pervanadate, were applied to each NHS-activated sepharose-bound WT or SsoPTP-C96S/D69A or beads alone. The three mixtures were incubated overnight at 4°C with continual rotation. Both bead-bound proteins and beads alone were intensively washed with buffer containing 150 mM NaCl to reduce non-specific protein-protein or beads-protein binding. Each slurry was then applied to a filter column, and further washed with 10 ml buffer containing 150 mM NaCl. Bound proteins were eluted from columns with a step gradient of NaCl (0.5M, 1M, and 1.5M). The eluate from the columns were analyzed by SDS-PAGE, followed by mass spectral analysis of specific bands present at the 0.5M, 1M, and 1.5M NaCl elution steps (Figure 6-2).

Analysis of eluted proteins by mass spectroscopy

As shown in Figure 6-2, six bands from the 1.5M NaCl elution step in SsoPTP-C96S/D69A lane were submitted to mass spectral analysis as tryptic peptides. These bands were identified as follows (from top to bottom) (Table 6-1): AAA ATPase (sso0421), pyruvate kinase (sso0981), hypothetical protein (sso1491), proteosome subunit α (sso0738), HSP20 (sso2427), and SsoPTP (detached from resin). Identification of *S. solfataricus* proteins were also performed at the 1M NaCl elution step from both WT and CD/DA lanes. Protein IDs were identical from the 1M NaCl and 1.5M NaCl elution steps. No protein were present in eluates from the 1M and 1.5M NaCl elution steps from the resin alone column, which may indicate that non-specific protein interaction with the resin can be eliminated at the 0.5M NaCl elution step.

The presence of *S. solfataricus* proteins in the wild type lane raised the possibility that those proteins might be associating with SsoPTP by protein-protein interaction rather than by the ability of inactive SsoPTP protein variant to bind to phosphorylated proteins. To assess this possibility, a recombinant form of either ssoHSP20 or ssoATPase or ssoPyK (pyruvate kinase) can be cloned and expressed in *E. coli* with a C-terminal hexahistidine tag for IMAC purification. A native gel electrophoresis and/or gel filtration chromatography can be used to determine whether SsoPTP wild type and ssoHSP20 or ssoATPase or ssoPyK can form a complex *in vitro*.

Proteasome subunit β and pyruvate kinase were shown to be phosphorylated on serine/threonine residues in the archaeon *Halobacterium salinarum* R1 by mass spectral analysis (Aivaliotis *et al.* 2009). Therefore, there is a possibility that *S. solfataricus* proteasome subunit α (archaeal proteasome consists of α and β subunits) and pyruvate kinase are phosphorylated on serine/threonine residues, and might constitute potential substrates since SsoPTP possess dual-specific characteristics *in vitro*. In addition, phosphorylation on serine/threonine residues can explain the negative results obtained with the Western blot analysis using anti-phosphotyrosine antibodies. Mass spectral analysis targeting phosphopeptides from proteasome subunit α and pyruvate kinase or possibly the other identified proteins should follow.

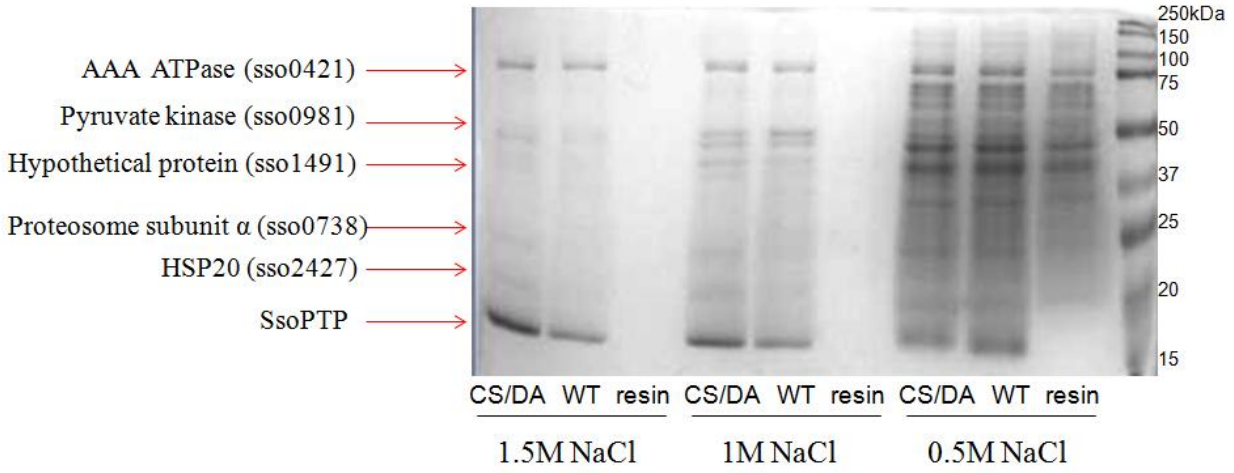


Figure 6-2. Isolation of endogenous proteins from *S. solfataricus* by *in vitro* substrate trapping pulldown. Coomassie blue staining of 12% SDS-PAGE gel of 400 µl TCA-precipitated *S. solfataricus* eluates from beads alone column (lanes labeled resin) or SsoPTP wild type column (lanes labeled WT) or substrate-trapping protein variant (SsoPTP-C96S/D69A) column (lanes labeled CS/DA) at 0.5M, 1M, and 1.5M NaCl elution steps, respectively. The bands present in the CS/DA lane at 1.5M NaCl elution step were identified by mass spectral analysis. The identification of each band is indicated on the far left with pointed red arrows. The last band is SsoPTP with hexahistidine tag, which was detached from the resin. The criteria for identifying each protein using mass spectral analysis are listed in Table 6-1 (for detailed description, refer to Materials and Methods, pg. 57). The pulldown experiment was repeated three times.

Protein name	ORF	NCBI accession number	Predicted MW (kDa)	Observed MW (kDa)	Mascot protein score	Sequence coverage (%)	Best individual ion score
AAA ATPase family protein	*sso0421*	gi|15897351	86	80	353	11	106
pyruvate kinase (pyK)	*sso0981*	gi|15897860	50	50	56	6	41
hypothetical protein	*sso1491*	gi|15898322	43	42	121	10	49
proteasome subunit alpha	*sso0738*	gi|15897639	27	25	58	8	34
hsp 20 family small heat shock protein	*sso2427*	gi|15899176	20	19	135	19	68
SsoPTP	*sso2453*	gi|145579198	18	18	419	45	156

Table 6-1. MALDI-TOF/TOF MS/MS identified *S. solfataricus* proteins from the 1.5M NaCl elution step in SsoPTP-C96S/D69A lane.

CHAPTER VII

Discussion

To date, two conventional protein tyrosine phosphates from two archaeal species have been characterized at the molecular level: *Tk*-PTP from *T. kodakaraensis* KOD1 (Jeon *et al.* 2002), and SsoPTP from *S. solfataricus* (this study). The primary amino acid sequences of both *Tk*-PTP and SsoPTP conform to the cPTP paradigm (HCX_5R).

Several eukaryotic and bacterial cPTPs have been well studied and their physiological roles have been established (PTP1B, TcPTP, etc), whereas insights into the physiological role of archaeal cPTPs remain fragmentary. Among the unanswered questions: whether eukaryotic and bacterial members of the cPTP family share similar biochemical characteristics and physiological roles as their archaeal counterparts or if the latter are imbued with distinct features and/or roles (if they are evolutionarily discrete). Did all members of the cPTP family descend from a common ancient family? In order to elucidate these questions, more biochemical and molecular characterizations of protein phosphatases from the three domains of life are needed.

The genome of *S. solfataricus* encodes a single conventional protein tyrosine phosphatase, SsoPTP, in addition to one protein serine/threonine phosphatases (PPPs) and ten 'eukaryotic-like' kinases (Kennelly 2003). The objective of this study was to determine whether SsoPTP possess similar characteristics in terms of its substrate specificity and chemical mechanism as the established members of the cPTP family, and to attain some insights into its physiological role by identifying possible SsoPTP substrates endogenous to *S. solfataricus*.

SsoPTP displayed the ability to dephosphorylate protein-bound phosphotyrosine as well as protein-bound phosphoserine/phosphothreonine.

S. solfataricus ORF, *sso*2453, encoding SsoPTP was cloned and expressed in *E. coli* as a recombinant fusion protein with a C-terminal hexahistidine tag. SsoPTP displayed phosphohydrolase activity toward *para*-nitrophenyl phosphate (*p*NPP), a common substrate for phosphohydrolases. The kinetic parameters of SsoPTP toward *p*NPP were similar to the kinetic parameters of known eukaryotic (VHR, PTP1B) and bacterial cPTPs (*Yersinia* PTP), implying that SsoPTP follows similar catalysis toward *p*NPP as other members of the cPTP family.

SsoPTP exhibited protein phosphatase activity towards a series of exogenous [^{32}P] phosphoprotein substrates that were either phosphorylated on serine/threonine residues or tyrosine residues: ^{32}P-S/T-Casein, ^{32}P-Y-Casein, ^{32}P-S/T-MBP, ^{32}P-S/T-RCML, and ^{32}P-Y-RCML. SsoPTP dephosphorylated ^{32}P-S/T-Casein, ^{32}P-Y-Casein, and ^{32}P-Y-RCML with roughly equal efficiency at substrate concentrations of 0.1, 0.3, and 0.5 μM substrate. It was observed that SsoPTP hydrolyzed ^{32}P-S/T-MBP and ^{32}P-S/T-RCML at a lower rate compared to the abovementioned substrates. However, SsoPTP displayed catalytic activity toward all of the exogenous [^{32}P] phosphoprotein substrates; therefore, SsoPTP possesses dual-specific activity.

SsoPTP hydrolyzes both isomers of naphthyl phosphate, an indication of dual specificity

The catalytic activity of SsoPTP against α- and β-naphthyl phosphate was measured. The isomers of naphthyl phosphate can give a rough estimate to the depth of the active site pocket of protein tyrosine phosphatases. X-ray crystallography studies revealed that the active site pocket of DSPs can accommodate phosphotyrosines as well as phosphoserine and phosphothreonine,

whereas, the active site pocket of strict PTPs can only accommodate phosphotyrosine. The structure of β-naphthyl phosphate resembles that of phosphotyrosine; hence, both DSPs and strict PTPs can dephosphorylate the β- isomer with roughly equal efficiency. The structure of α-naphthyl phosphate, on the other hand, mimic the structure of either phosphothreonine or phosphoserine, and thus the α-isomer can only be accessed by the active site of DSPs.

SsoPTP displayed phosphohydrolase activity toward both isomers of naphthyl phosphate with 1 to 1.5 ratio of α to β. This indicates that the active site pocket of SsoPTP is similar to the active site pockets of DSPs – wide and shallow. Therefore, SsoPTP is a dual-specific phosphatase *in vitro*, which is also consistent with the ability of SsoPTP to dephosphorylate protein-bound phosphotyrosine as well as protein-bound phosphoserine/phosphothreonine.

The four conserved residues within the presumed active site sequence: Asp^{69}, His^{95}, Cys^{96}, and Arg^{102}, and the invariant Gln^{139} residue are essential for catalysis

Site-directed mutagenesis was performed on SsoPTP to selectively alter residues within the active site sequence that were shown to affect catalysis. Mutagenically altered forms of SsoPTP with either a D69A, H95A, C96S or R102A(K) substitution displayed little to no catalytic activity when assayed with *p*NPP. This result is consistent with mutagenic alterations of the same residues in other eukaryotic and bacterial cPTP (e.g. PTP1B and *Yersinia* PTP), which implies that the catalytic mechanism of SsoPTP parallels that of the established members of the cPTP family that involves the formation of phosphocysteinyl enzyme intermediate.

In addition to the conserved residues within the active site sequence, the substitution of the invariant Gln^{139} to Ala abolished the catalytic activity of SsoPTP. This result may represent a

new finding since there is no report of other cPTPs that are catalytically impaired as a result of the alteration of this same Gln. However, alteration of another invariant Gln residue (Gln^{135} in SsoPTP) was shown to dramatically decrease the catalytic activity of PTP1B (Gln^{262}) and *Yersinia* PTP (Gln^{446}). Both primary amino acid and active site 3-dimensional alignment of SsoPTP with PTP1B and *Yersinia* PTP revealed that Gln^{135} of SsoPTP closely aligns with both Gln^{262} in PTP1B and Gln^{446} in *Yersinia* PTP, which were shown to abolish activity of the enzyme. Hence, the location of the essential Gln^{139} in SsoPTP is different in PTP1B and *Yersinia* PTP. However, it might be premature to conclude that this Gln^{139} residue is essential for catalysis solely in archaeal PTPs (or SsoPTP) because of the limited number of mutagenic studies on the two Gln residues thus far (one eukaryotic: PTP1B, one bacterial: *Yersinia* PTP and one archaeal: SsoPTP).

Chemical rescue by exogenous guanidine of the Arg102Ala form of SsoPTP

Chemical rescue approach has been used to show whether a residue is essential for catalysis by enhancing or restoring the activity of a catalytically impaired enzyme in which one or more residues have been changed, with an exogenous compound that can mimic and compensate for the side chain of the residue(s) that has been altered. Exogenous guanidine hydrochloride enhanced the activity of SsoPTP-R102A by 2.4 fold when assayed with *p*NPP. The side chain of Arg^{102}, the guanidino group, can form salt bridges with the phosphate group on the substrate, which results in stabilization of the transition state. Guanidine hydrochloride can mimic guanidino group on Arg^{102} and partially stabilize the transition state. However, guanidine hydrochloride did not rescue the activity of SsoPTP-R120A to a wild type rate. This could be due to the limited access of guanidine hydrochloride compounds to the cavity of the active site of

the enzyme because of ionic interaction within the active site. In addition, guanidine hydrochloride must be oriented correctly in order to interact with the substrate, which can be difficult due to their free movement within the 3-dimensional space of the active site (unlike the covalently attached guanidino group of Arg^{102}).

Several other rescue agents were used in an attempt to rescue/enhance the catalytic activity of SsoPTP protein variants. However, none of these agents were able to enhance the catalytic activity to detectable levels. One possible reason is the accessibility of rescue agents to the active site of the enzyme, where the altered residue resides. The limited accessibility can be caused by same charge repulsion between residues within or at the entrance of the active site and the charged rescue agents, or by steric hindrance of adjacent residues occupying the cavity of the active site.

Loss of activity was caused by altering essential catalytic residues rather than a misfolded protein

Partial tryptic digestion was employed to detect if altering residues within the active site of SsoPTP caused conformational changes in the protein. Trypsin hydrolyzed the wild type enzyme and its protein variants with equal rate. The rate of appearance of the bands of the digested proteins on an SDS-PAGE gel was roughly the same with similar pattern of fragments produced. This indicates that all SsoPTP protein variants fold similarly to the wild type enzyme, and therefore, loss of activity was caused by altering essential residues that effect catalysis.

Several endogenous proteins from *S. solfataricus* were isolated by *in vitro* substrate trapping pulldown

Substrate trapping or pulldown utilizes a catalytically impaired enzyme to bind potential substrates. This catalytically impaired protein variant is produced by altering the active site cysteine to serine residue or altering both cysteine and the essential aspartic acid residue to serine and alanine, respectively. The latter protein variant has been shown to have a higher affinity toward binding its substrates, and therefore serves as a better substrate trapping protein variant (Xie *et al.* 2002). SsoPTP-C96S/D69A was constructed to trap possible substrates present in lysates of *S. solfataricus*, pretreated with 1 mM PTP inhibitor, pervanadate, which can also cause oxidative stress. This treatment was done to enrich for protein phosphorylation in *S. solfataricus*.

A concentration step gradient of NaCl was employed to elute proteins that adhered to columns containing either NHS-activated Sepharose bead-bound wild type or protein variant or resine alone. The column containing resin alone (no ligand attached) was used as a negative control to identify proteins that non-specifically interacted with the resin. At 0.5M NaCl, it was observed that several *S. solfataricus* proteins were present in the resin alone column, when a sample of the eluate was run on an SDS-PAGE gel. There were no *S. solfataricus* proteins in the resin alone column at the 1M and 1.5M NaCl; therefore, most of non-specific interaction of protein with the resin can be eliminated at the 0.5M salt elution step. Any *S. solfataricus* proteins that can survive the higher salt elution steps may indicate stronger association with SsoPTP wild type or protein variant, and thus, a possible potential substrate or interacting protein with SsoPTP can be detected.

Six bands were detected in the wild type and protein variant lanes at the 1M and 1.5M salt elution steps as observed on an SDS-PAGE gel. Mass spectral analyses were performed on the six bands present at the 1M and 1.5M salt elution step in both protein variant and wild type lanes. All the identified bands were identical in the wild type and protein variant lanes at the 1M and 1.5M salt elution steps. These bands are (from top to bottom): AAA ATPase (*sso0421*), pyruvate kinase (*sso0981*), hypothetical protein (*sso1491*), proteasome subunit α (*sso0738*), HSP20 (*sso2427*), and SsoPTP (detached from resin).

S. solfataricus AAA ATPase (*sso0421*) belongs to the AAA ATPase family (ATPase Associated with various cellular Activities) that is conserved in eukaryotes, bacteria, and archaea. In eukaryotes, AAA ATPases are involved in several cellular processes including cell cycle, DNA replication and repair, protein folding, and proteolysis (Smith *et al.* 2006). It was shown in eukaryotes that the AAA ATPase associates with the 26S proteasome complex for protein degradation in an ATP-dependent process. The eukaryotic AAA ATPase forms a hexameric ring-like structure with a central pore in which protein folding or unfolding occurs. Six homologous subunits of AAA ATPase form the cap of the 26S proteasome complex in which unfolded proteins translocate from the central pore of the ATPase into the proteasome complex (White *et al.* 2007).

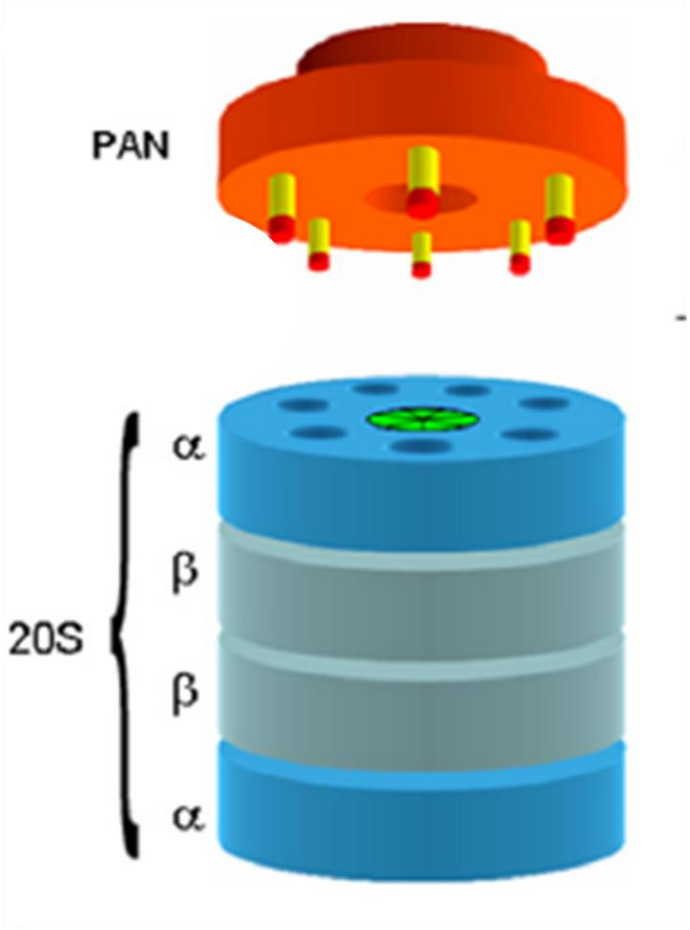
In the *Archaea,* AAA ATPase was shown to interact with the archaeal 20S proteasome complex as was seen in eukaryotes. A gene (*s4*) from *Methanococcus jannaschii* encodes a 50 kDa protein, named PAN (proteasome-activating nucleotidase), that was shown to share 41–45% sequence similarity with the eukaryotic 26S AAA ATPase. PAN assembles into a hexameric ring-like structure that forms a cap on the 20S proteasome complex which consists of 2 α and 2 β subunits as shown in Figure 7-1 (Smith *et al.* 2006).

There have been no studies on the *S. solfataricus* AAA ATPase (*sso0421*) so far. However, it can be hypothesized to have a chaperone-like function that involves association with the *S. solfataricus* proteasome complex. Interestingly, a proteasome subunit α (*sso0738*) was also trapped by SsoPTP protein pulldown experiment. In the model shown in Figure 1, AAA ATPase interacts with the α subunit of the 20S proteasome; therefore, *S. solfataricus* AAA ATPase might also form a complex with the proteasome α subunit. Furthermore, mass spectral analysis showed that the proteasome β subunit of *H. salinarum* is phosphorylated (Aivaliotis *et al.* 2009). Hence, it is possible that the proteasome α subunit of *S. solfataricus* be phosphorylated, and therefore, SsoPTP may dephosphorylate this subunit causing the proteasome to assemble with the AAA ATPase for protein degradation in a stress-induced response caused by pervanadate.

In addition, the small heat shock protein HSP20 (*sso2427*) is predicted to interact with AAA ATPase (*sso0421*) by the STRING database (Jensen *et al.* 2009) as shown in Figure 7-2. This database combines information on protein-protein interactions from experiments, computational predictions, and available literature, which covers about 2.5 million proteins from 630 organisms (Jensen *et al.* 2009). The expression of HSP20 was shown to be upregulated in response to H_2O_2 in *S. solfataricus* (Maaty *et al.* 2009). Therefore, SsoPTP might be a part of a protein complex of chaperones to regulate either their function or downstream proteins.

Mass spectral analysis on pyruvate kinase from the archaeon *H. salinarum* revealed that it is phosphorylated on serine/threonine residues (Aivaliotis *et al.* 2009). If pyruvate kinase from *S. solfataricus* can also be phosphorylated, there may be a link between SsoPTP and pyruvate kinase in which the latter constitutes a substrate for SsoPTP or form a complex with it, since *S. solfataricus* proteins were also present in the wild type lane as well as the SsoPTP-C96S/D69A lane from the pulldown experiment outlined above.

To determine if SsoPTP can form a complex with one or more of the proteins mentioned above, an *in vitro* experiment can be employed where recombinant SsoPTP can be incubated with either recombinant AAA ATPase or HSP20 or pyruvate kinase or proteasome α subunit. Size exclusion chromatography or native gel electrophoresis can be used to determine the presence of such a complex.



[fair use]

Figure 7-1. PAN association with the archaeal 20S proteasome. PAN is shown in red, the two α subunits of the 20S proteasome are shown in blue, the β subunits are shown in cyan. The central core of the 20S proteasome is depicted in yellow (Smith *et al.* 2006).

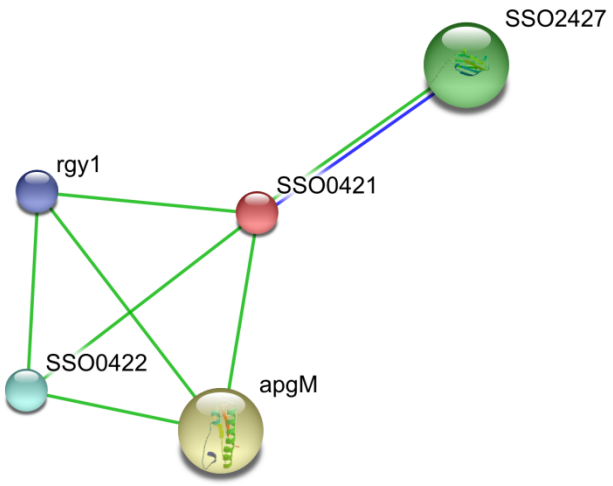


Figure 7-2. Predicted interactions of HSP20 (sso2427) and AAA ATPase (sso0421) by STRING (Known and Predicted Protein-Protein Interactions Database). The predicted functional partners are: apgM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, sso2427, Small heat shock protein hsp20 family (176 aa), sso0422, Putative uncharacterized protein (293 aa), rgy1, reverse gyrase 1. The green lines indicates interactions based on neighboring prediction, whereas the blue line between AAA ATPase and HSP20 indicates co-occurance in various species. The crystal structure of HSP20 and apgM are shown in their corresponding spheres.

Conclusions

ORF *sso2453*, from the acidothermophilic archaeon, *Sulfolobus solfataricus*, encodes an 18 kDa protein with a predicted phosphatase activity owing to the presence of the conventional protein tyrosine phosphatase family, cPTP, signature motif, $DX_{27}HCX_5R$. A recombinant form of this protein, SsoPTP, was cloned and expressed in *E.coli* with a C-terminal hexahistidine tag to simplify its purification. SsoPTP displayed phosphohydrolase activity when challenged with *p*NPP, a common substrate for phosphohydrolases. Its catalytic activity was comparable to other members of the cPTP family, indicating that SsoPTP is indeed a phosphatase.

SsoPTP was able to dephosphorylate both ^{32}P-labeled protein-bound phosphoserine/threonine and ^{32}P-labeled protein-bound phosphotyrosine. Furthermore, SsoPTP dephosphorylated α- and β-naphthyl phosphate with equal efficiency. Collectively, SsoPTP displays dual-specific phosphatase activity *in vitro.*

The chemical mechanism of SsoPTP was observed to be similar to the proposed mechanism of established members of the cPTP family since altering essential active site residues abolished catalytic activity as predicted.

Several candidate protein substrates were isolated from *S. solfataricus* cell lysates by SsoPTP substrate trapping pulldown. The identity of each isolated protein was determined by mass spectroscopy as follows: AAA ATPase (*sso0421*), pyruvate kinase (*sso0981*), hypothetical protein (*sso1491*), proteasome subunit α (*sso0738*) and HSP20 (*sso2427*). The relationship of each protein with SsoPTP is to be determined.

Future directions

Substrate trapping has many limitations – low cellular concentrations of the endogenous target proteins to be trapped, changes in affinity of the enzyme to its substrates in an *in vitro* setting, inefficient interactions with potential substrates due to coupling of an enzyme to a resin, and possible transient association of catalytically impaired enzyme with its substrates. In addition, several false-positives can also be trapped. Therefore, an alternative experiment can be performed with an *in vivo* approach. *S. solfataricus* cells can be transformed with exogenous DNA by electroporation. By this means, an inducible-vector containing SsoPTP-C96S/D69A gene can be transformed into *S. solfataricus* competent cells (Jonuscheit *et al.* 2003; Albers *et al.* 2006). Cells can then be induced for over-expression of SsoPTP-C96S/D69A with a tag for purification. Hence, SsoPTP-C96S/D69A can be purified from lysates of *S. solfataricus* cells through the interaction of the tag with its corresponding resin (Blanchetot *et al.* 2005). SsoPTP-C96S/D69A and any bound *S. solfataricus* proteins can then be eluted from the column. The proteins can be resolved by SDS-PAGE followed by mass spectral analysis. A control of the wild type enzyme should also be performed in parallel with the protein variant co-precipitation experiment.

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Chapter VIII

A PPM-family protein phosphatase from the thermoacidophile *Thermoplasma volcanium* hydrolyzes protein-bound phosphotyrosine

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Ben Potters and I were the major contributors to the experiments mentioned in this paper, including cloning of ORF *tvn0703* and expression of its recombinant protein product in *E. coli*, protein purification, and assays of phosphatase activity. I helped with the preparation of the manuscript by writing a portion of the Materials and Methods, and preparing figures and tables. I also generated the phylogenetic tree of TvnPPM described in this paper. AbdulShakur Abdullah contributed to the initial characterization of TvnPPM. My advisor, Dr. Peter Kennelly, supervised the entire work described in this paper and prepared the manuscript.

Keywords: Protein phosphorylation, Signal transduction, Dual-specific protein phosphatase, Archaea, Protein-tyrosine phosphatase, Protein-serine/threonine phosphatase, Zinc

Abstract

The genomes of virtually all free-living archaeons encode one or more deduced protein-serine/threonine/tyrosine kinases belonging to the so-called eukaryotic protein kinase superfamily. However, the distribution of their cognate protein-serine/threonine/phosphatases displays a mosaic pattern. *Thermoplasma volcanium* is unique among the *Archaea* inasmuch as it is the sole archaeon whose complement of deduced phosphoprotein phosphatases includes a member of the PPM-family of proteinserine/threonine phosphatases—a family that originated in the *Eucarya*. A recombinant version of this protein, TvnPPM, exhibited protein-tyrosine phosphatase in addition to its predicted protein-serine/threonine phosphatase activity *in vitro*. TvnPPM is the fourth member of the PPM-family shown to exhibit such dual-specific capability, suggesting that the ancestral versions of this enzyme exhibited broad substrate specificity. Unlike most other archeaons, the genome of *T. volcanium* lacks open reading frames encoding stereotypical protein-tyrosine phosphatases. Hence, the dual-specificity of TvnPPM may account for its seemingly aberrant presence in an archaeon.

Introduction

The physicochemical properties of the phosphoryl group render it an exceptionally potent agent for perturbing protein structure (Johnson and Lewis 2001; Kennelly 2008). Consequently, the reversible modification of proteins by covalent phosphorylation and dephosphorylation has evolved into nature's most capable and versatile mechanism for modulating protein function, transmitting molecular signals, and processing sensory information. Critical to the emergence of protein phosphorylationdephosphorylation as a molecular regulatory mechanism was the development of two classes of specialized signal transmission enzymes: protein kinases to catalyze the addition of phosphoryl groups to proteins and protein phosphatases to restore

phosphoproteins to their unmodified starting state. Our laboratory has been investigating the protein kinases, protein phosphatases, and phosphoproteins resident within the *Archaea* in an effort to pinpoint the origins and retrace the development of this important molecular regulatory paradigm.

Open reading frames (ORFS) encoding known or deduced members of the so-called eukaryotic protein kinase (ePK) superfamily are ubiquitous among the *Archaea* (D'Souza et al. 2007). As its name suggests, the members of this superfamily of protein-serine/threonine/tyrosine kinases for a long time were considered to be eucaryal in origin and distribution (Kennelly and Potts 1996). However, the new, global perspective provided by genomics has since revealed that the ePK superfamily appeared long ago—prior to the divergence of the *Eucarya* from the *Archaea* (Leonard et al. 1998; Kannan et al. 2007).

In contrast to the ePKs, the distribution of countervailing protein phosphatases appears strikingly irregular and inconsistent (Kennelly 2003; Bhaduri and Sowdhamini 2005). Surprisingly, the majority of archaeal genomes encode one or more deduced protein-tyrosine phosphatases (PTPs) (Kennelly 2003; Bhaduri and Sowdhamini 2005). In fact, many archeaons contain only deduced PTPs—either conventional PTPs, low molecular weight PTPs, or a combination thereof—in their suite of potential protein-serine/threonine/tyrosine phosphatases. Intriguingly, while many archaeons contain PPP-family protein-serine/threonine phosphatases, only one ORF encoding a deduced PPM-family (Barford 1996) protein-serine/threonine phosphatase has been encountered: TVN0703 from the acidothermophile *Thermoplasma volcanium* (Kennelly 2003; Bhaduri and Sowdhamini 2005). In this paper we ask whether TvnPPM, the unique protein encoded by ORF TVN0703, possesses protein-serine/threonine/tyrosine phosphatase activity.

Materials and methods

Materials

Purchased materials included mixed histones type II-AS, a 5% (w/v) solution of partially dephosphorylated casein, myelin basic protein (MBP), the catalytic subunit of the cAMP-dependent protein kinase, and *p*-nitrophenyl phosphate (Sigma, St Louis, MO); *Pfu* Turbo DNA Polymerase, BL21-CodonPlus (DE3)-RIL cells, and a Quik-Change II site-directed mutagenesis kit (Stratagene; LaJolla, CA); QIAquick PCR purification and QIAprep spin miniprep kits (Qiagen; Valencia, CA); expression vector pET-29b, S-protein agarose, and an S Tag Thrombin Purification kit (Novagen; San Diego, CA). All oligonucleotides were from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Genomic DNA from *T. volcanium* was from the American Type Culture Collection (Manassas, VA). [γ-^{32}P] ATP was from Perkin Elmer Life Sciences (Beverly, MA). *Escherichia coli* expressing the *Lyn* protein-tyrosine kinase as a GST fusion protein was the gift of Profs. Marietta Harrison and Harry Charbonneau of Purdue University (West Lafayette, IN). All other reagents were from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Routine procedures

Protein concentrations were determined as described by Bradford (1976) using premixed reagent and a standardized solution of bovine serum albumin from Pierce (Rockford, IL). SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue as described by Fairbanks et al. (1971).

Cloning of TVN0703 and expression of its protein product in *E. coli*

ORF TVN0703 was amplified by PCR using genomic DNA from *T. acidophilum* as template essentially as described by Li et al. (2005) using the following forward and reverse primers, respectively: 5′-TTGATGGCCATGGTAAAAGTTGAATATTTTTCAGATTCCAG-3′ and 5′-CACAGCGTCGACTTACGCCATTGTAAC-3′. These primers introduced overhanging sequences complementary to the restriction sites generated by *NcoI* or *SalI*. The resulting PCR product was ligated into expression vector pET-29b and the resulting plasmid used to transform One Shot TOP 10 chemically competent *E. coli*. The orientation and sequence of the cloned gene was verified by sequencing the isolated plasmid. For recombinant protein expression, the plasmid was used to transform *E. coli* BL21-CodonPlus (DE3)-RIL competent cells. The transformed cells were cultured at 37°C in 250 ml of Luria–Bertani medium containing 0.1 mg/ml kanamycin, 34 µg/µl chloramphenicol until the OD_{600} fell within the range of 0.6–1.0. Isopropyl-β-D-thiogalactopyranoside then was added to a final concentration of 1 mM and the culture incubated for another 3 h. Cells were then harvested by centrifugation, lysed by sonic disruption, and cell extracts prepared by standard procedures (Li et al. 2005). Recombinant protein was purified using S-protein agarose and the fusion domain subsequently removed using an S Tag Thrombin Purification kit, both as described by the manufacturer.

Assay of phosphatase activity

Protein phosphatase activity was assayed using a modification of the procedure of McGowan and Cohen (1988). Briefly, 1.0–1.5 µg recombinant TvnPPM was incubated at

55°C in a volume of 50 µl containing 100 mM Tris–HCl, pH 8.5, 1 mM $MnCl_2$, 0.2 mg/ml bovine serum albumin, and, unless indicated otherwise, a phosphoprotein substrate at a concentration 1.2 µM protein-bound [^{32}P] phosphate. Phosphoprotein substrates were phosphorylated on serine and threonine residues using [γ-^{32}P] ATP and the catalytic subunit of cAMP-dependent protein kinase, or on tyrosine residues using [γ-^{32}P] ATP and the *lyn* protein-tyrosine kinase, as described in (Kennelly et al. 1993). The reaction was terminated, typically after an incubation period of 15 min, by the addition of 150 µl of 20% (w/v) trichloroacetic acid. After agitating briefly using a Vortex mixer, the mixture was centrifuged for 3 min at 12, 000 x *g*. A portion, 50 µl, of the supernatant liquid was removed and counted for ^{32}P radioactivity in 2.0 ml of Scintisafe 30% liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA). Where indicated, the source of the ^{32}P-labeled material in the TCA supernatant was verified as being inorganic phosphate via extraction into organic solvents as a molybdate complex by the procedure of Martin and Doty (1949).

Activity toward *p*-nitrophenyl phosphate (*p*NPP) was assayed under conditions similar to those described above for phosphoprotein substrates with the exception that the volume of the initial incubation was increased to 250 µl and no BSA was added. The concentration of *p*NPP was 10 mM. The reaction was terminated by the addition of 750 µl of 0.5 M EDTA, pH 10. *p*-Nitrophenolate was then measured spectrophotometrically at a wavelength of 410 nm.

Results

TvnPPM exhibits divalent metal ion-dependent phosphatase activity

While only 218 residues in length, TvnPPM from *T. volcanium* contains all of the signature motifs conserved among the PPM family of protein phosphatases (Figure 8-1). The M in PPM refers to the universal dependence of these enzymes on the presence of divalent metal ions such as Mg^{2+} or Mn^{2+} for catalytic activity, one of the functional characteristics by which they were originally distinguished from the other major family of protein-serine/threonine phosphatases, the PPP family (Barford 1996). TvnPPM was expressed as a recombinant fusion protein in *E. coli*, then incubated with thrombin to remove the fusion domain. When challenged with *p*-nitrophenyl phosphate, a general purpose substrate for phosphohydrolases, TvnPPM exhibited divalent metal ion-dependent phosphatase activity. Of the potential cofactors tested, Mg^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}, only the last named supported catalysis on its own. As the metal centers in PPM-family protein phosphatases are di- (Das et al. 1996; Bellinzoni et al. 2007) or, in some cases, tri- (Pullen et al. 2004; Rantanen et al. 2007; Wehenkel et al. 2007; Schlicker et al. 2008) nuclear, we asked whether adding a second metal ion in addition to Mn^{2+} would yield greater catalytic activity than that obtained using Mn^{2+} alone.

While the inclusion of Mg^{2+}, Ni^{2+}, Ca^{2+}, and Cu^{2+} along with Mn^{2+} had little effect on activity relative to that observed with Mn^{2+} alone, Zn^{2+} inhibited the enzyme in a concentration-dependent manner (Figure 8-2). Inhibition by Zn^{2+} was irreversible. The removal of free Zn^{2+} via the addition of EDTA or by extensive dialysis versus EDTA-containing buffers failed to restore any trace of Mn^{2+}-dependent catalytic activity to TvnPPM. Attempts to displace any bound zinc by prolonged dialysis versus Mn^{2+}-containing buffers also had failed to restore activity. While

protein phosphatase 2Cα from humans, an eukaryotic PPM, also is inhibited by Zn^{2+}, kinetic analyses revealed that zinc acted via a reversible, competitive mechanism versus metal ion cofactors (Fjeld and Denu 1999). Presumably, the binding of Zn^{2+} to TvnPPM triggers a conformational transition analogous to that by which this metal ion irreversibly inhibits alkaline phosphatase from the green crab Scylla serrata (Zhang et al. 2001).

TvnPPM exhibits dual-specific protein phosphatase activity *in vitro*

TvnPPM was challenged with a series of radiolabeled phosphoproteins. As is typical of other members of the PPM family, TvnPPM dephosphorylated casein and reduced, carboxyamidomethylated and maleylated (RCM) lysozyme that had been phosphorylated on serine and threonine residues using the catalytic subunit of the cAMP-dependent protein kinase (P-Ser/Thr casein and P-Ser/Thr RCM lysozyme) (Table 8-1). The reaction product could be extracted into organic solvents as a molybdate complex, unambiguously establishing its identity as inorganic phosphate (Data not shown). TvnPPM is not completely promiscuous, however, as it displayed little activity toward P-Ser/Thr histones.

The limited number and heterogeneous distribution of deduced protein-serine/threonine/tyrosine phosphatases in the *Archaea* (Kennelly 2003) suggests that these enzymes may—of necessity—be more versatile than their eucaryal counterparts. For example, in those archaeons that possess only canonical PTPs, it is quite likely that these enzymes function as dual-specific, i.e. protein-serine/threonine as well as protein-tyrosine, phosphatases. The converse apparently does not, however, hold for archaeal PPP-family protein phosphatases. All archaeal PPPs characterized to date are strictly serine/threonine-specific (Kennelly et al.

1993; Leng et al. 1995; Solow et al. 1997; Mai et al. 1998), consistent with their high sequence identity to eucaryal PPPs, which are serine/threonine-specific. *T. volcanium* is unusual inasmuch as it does not possess any deduced PTPs, only TvnPPM and a deduced PPP (Kawashima et al. 2000). We therefore asked whether, in *T. volcanium*, TvnPPM fulfills the catalytic function(s) normally supplied in other archaeons by either conventional or low molecular weight PTPs. If so, we would expect that TvnPPM would exhibit dual-specific protein phosphatase activity.

TvnPPM was challenged with several phosphotyrosine-containing [P-Tyr] proteins that had been prepared using [γ-^{32}P] ATP and the *lyn* protein-tyrosine kinase *in vitro*: P-Tyr casein, P-Tyr RCM lysozyme, and P-Tyr MBP. As can be seen in Table 8-1, TvnPPM dephosphorylated all three phosphoproteins at rates comparable in magnitude to that at which it dephosphorylated P-Ser/Thr casein and P-Ser/Thr RCM lysozyme, and $\approx$3–6 fold greater than P-Ser/Thr histones.

Phylogenetic position of TvnPPM

Phylogenetic trees show TvnPPM to be very deeply rooted (Figure 8-3), suggesting that *T. volcanium* acquired the gene encoding this protein phosphatase at a relatively early stage in the evolution of the PPM family. This supposition is reinforced by the close association between TvnPPM with AaPPM, a PPM-family protein phosphatase from the bacterium *Aquifex aeolicus* (Purcarea et al. 2008). *A. aeolicus* is a thermophile that occupies the lowest branch within the prokaryotic portion of the phylogenetic tree (Coenye and Vandamme 2004).

Discussion

TvnPPM ranks as one of the most distinctive members of the PPM family of protein phosphatases. While the PPM paradigm has long been considered to be serine/threoninespecific

(Barford 1996; Jackson and Denu 2001), TvnPPM exhibited significant protein-tyrosine phosphatase activity in vitro in addition to its predicted protein-serine/threonine phosphatase activity. Moreover, TvnPPM remains the sole archaeal representative of the PPM family (Bhaduri and Sowdhamini 2005). Since TvnPPM constitutes the only apparent source of protein-tyrosine phosphatase activity— or perhaps some other phosphatase activity for which tyrosine phosphatase activity is a manifestation—in *T. volcanium*, we suggest that its dual-specific activity and unique phylogenetic status are linked.

Is it reasonable to expect that *T. volcanium* requires a source of protein-tyrosine phosphatase activity? The fact that the majority of archaeal genomes encode one or more canonical PTPs, and that some possess only conventional and/or low molecular weight PTPs certainly suggests as much (Kennelly 2003; Bhaduri and Sowdhamini 2005). More importantly, proteins recognized by antibodies against phosphotyrosine have been detected in halophilic, methanogenic, as well as thermophilic archaeons (Smith et al. 1997; Jeon et al. 2002).

Is TvnPPM a bona fide dual-specific protein phosphatase? While the range of phosphoprotein substrates tested was constrained by the difficulties of generating them in reagent quantities, the enzyme's activity toward all three phosphotyrosine-containing proteins tested approached that exhibited toward P-Ser/Thr casein and P-Ser/Thr RCM lysozyme, and exceeded by several-fold its protein-serine/threonine phosphatase activity toward P-Ser/Thr histones. The magnitude of TvnPPM's protein-tyrosine phosphatase activity thus exceeds that attributable to non-specific ''background'' hydrolysis. Moreover, TvnPPM is not the first PPM to exhibit dual-specific capabilities *in vitro*. PphA (Ruppert et al. 2002) and SynPPM3 (Li et al. 2005) from the cyanobacterium *Synechocystis* sp. strain PCC6803 as well as PrpZ from *Salmonella enterica serovar Typhi* (Lai and Le Moual 2005) all reportedly possess significant protein-tyrosine

phosphatase activity. In addition, PP2C from rabbit exhibits protein histidine phosphatase activity *in vitro* (Kim et al. 1993). Taken together, it appears likely many PPMs, including TvnPPM, function as dual-specific protein phosphatases.

How did *T. volcanium* acquire TvnPPM? Phylogenetic trees constructed by our laboratory and others (Zhang and Shi 2004) indicate that TvnPPM is deeply rooted, suggesting that *T. volcanium* acquired the gene encoding this protein phosphatase at a relatively early stage in evolution. This supposition is reinforced by close association between TvnPPM and AaPPM from *A. aeolicus* (Purcarea et al. 2008), which resides near the root of the bacterial phylogenetic tree (Coenye and Vandamme 2004). While it is difficult to discern whether the *Archaea* acquired the gene for TvnPPM directly from the *Eucarya* or through a bacterial intermediary, the respective genetic contexts of TvnPPM and AaPPM favor the latter route. The gene for AaPPM overlaps that of its cognate protein kinase, AaSTPK, by one base pair, while the region surrounding the gene for TvnPPM is devoid of coding sequences for potential protein kinases. As the typical subfamily of ePKs to which AaSTPK belongs apparently arose in the *Eucarya* (Leonard et al. 1998) it is likely that both kinase and phosphatase were acquired together (Ponting et al. 1999). Subsequently, the gene for an AaPPM-like protein phosphatase found its way to a neighboring archaeon within an ancient community of thermophiles.

The early acquisition of TvnPPM by the domain *Archaea* begs the question, why didn't this protein phosphatase family spread to other members of the domain? It certainly was not for lack of opportunity. The environments favored by *T. volcanium* are inhabited by a variety of acidothermophiles, including numerous archaeons (Segerer et al. 1988; Simmons and Norris 2002; Wagner and Wiegel 2008), which engaged in an extensive exchange of genetic material (Boucher et al. 2003; Choi and Kim 2007). One possible explanation derives from the hypothesis

that broad segments of the archaeal population have undergone significant reductive evolution (Kurland et al. 2007; Wang et al. 2007). As the latecomer amongst the protein phosphatases in the *Archaea*, the forces tending to support the dissemination of this new signal transmission module likely could not withstand the countervailing tide of genomic downsizing. However, in the case of *T. volcanium*, circumstances conspired to cause its endogenous PTP(s) to be lost, leading to the retention of TvnPPM as substitute. If this conclusion is correct, when additional archaeal PPMs are eventually encountered, then the host archaeon will prove lacking genes encoding deduced PTPs.

Acknowledgments

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Figures and Tables

Figure 8-1. DNA-derived amino acid sequence of the protein product of ORF TVN0703. Shown is the amino acid sequence of TvnPPM, the protein product of ORF TVN0703 (GenBank Accession No. NP_111222) aligned against the sequence of AaPPM from Bacillus subtilis (GenBank Accession No. NP_213403). Matches are indicated by colons and similarities by periods. Below the protein sequences are shown the conserved subdomain sequences characteristic of the PPM family of protein phosphatases (Bork et al. 1996) as described using the nomenclature of Hanks and Hunter (1995): upper case letters conserved amino acids residues, lower case letters generally conserved amino acid residues, x any amino acid, o positions where non-polar residues are conserved, plus positions where small neutral residues are conserved, asterisk positions where polar residues are conserved, and ash positions where charged residues are conserved.

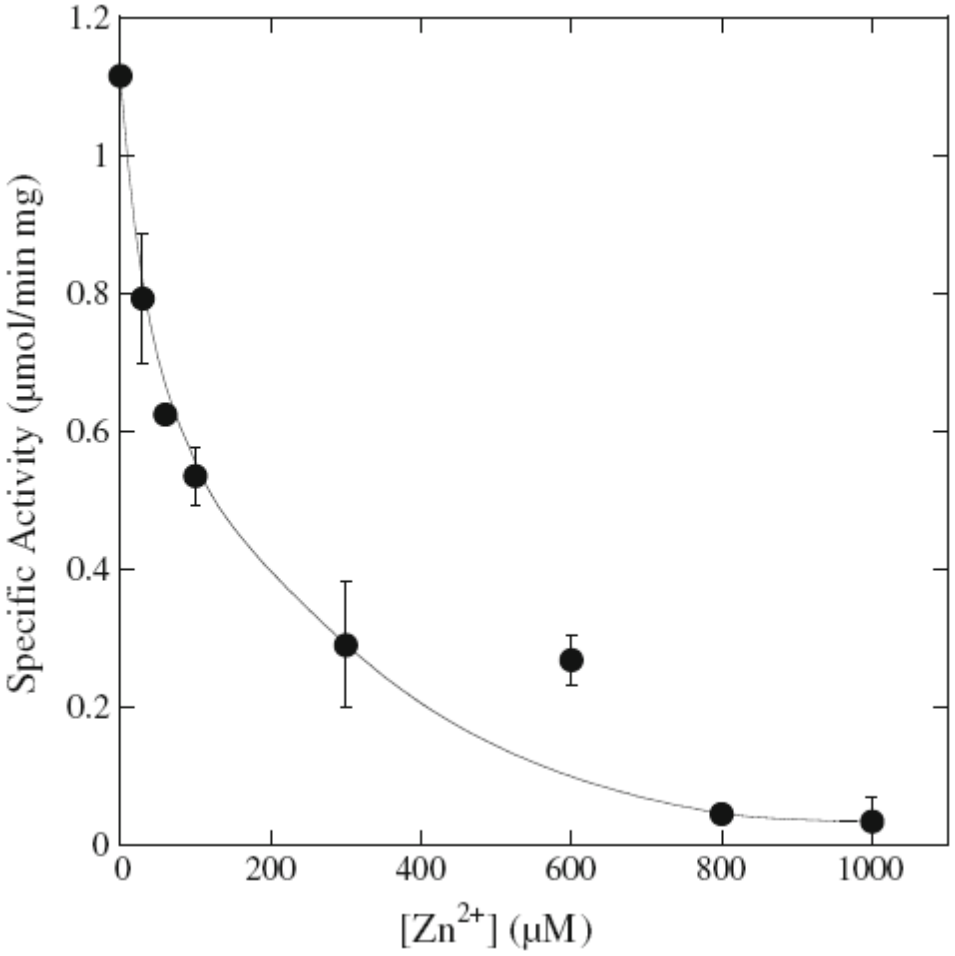

Figure 8-2. Zn^{2+} inhibits the activity of TvnPPM. The catalytic activity of TvnPPM, 0.5 µg, toward *p*NPP was assayed as described in ‘‘Materials and methods’’ with the exception that, where indicated, $ZnCl_2$ was added to the listed final concentration. Shown is a plot of specific activity versus Zn^{2+} concentration ± SE (n = 3). The curve shown was fitted using all points but one, that for 600 µM Zn^{2+}.

Substrate	Specific activity		
	pmol/ min mg	P-Ser/Thr casein (%)	P-Ser/Thr RCM (%)
Lysozyme			
P-Ser/Thr casein	160 ± 20	100	269
P-Ser/Thr histones	3 ± 1	2	5
P-Ser/Thr RCM lysozyme	59 ± 3	37	100
P-Tyr casein	15 ± 4	9	25
P-Tyr RCM lysozyme	8 ± 2	5	14
P-Tyr MBP	18 ± 2	11	31

Table 8-1. TvnPPM exhibits dual-specific protein phosphatase activity. The catalytic activity of TvnPPM, 1.5 µg, was assayed toward the listed phosphoprotein substrates as described in ‘‘Materials and Methods’’. Shown is the protein phosphatase activity toward each phosphoprotein ± SE (n = 2) as relative activity versus selected substrates.

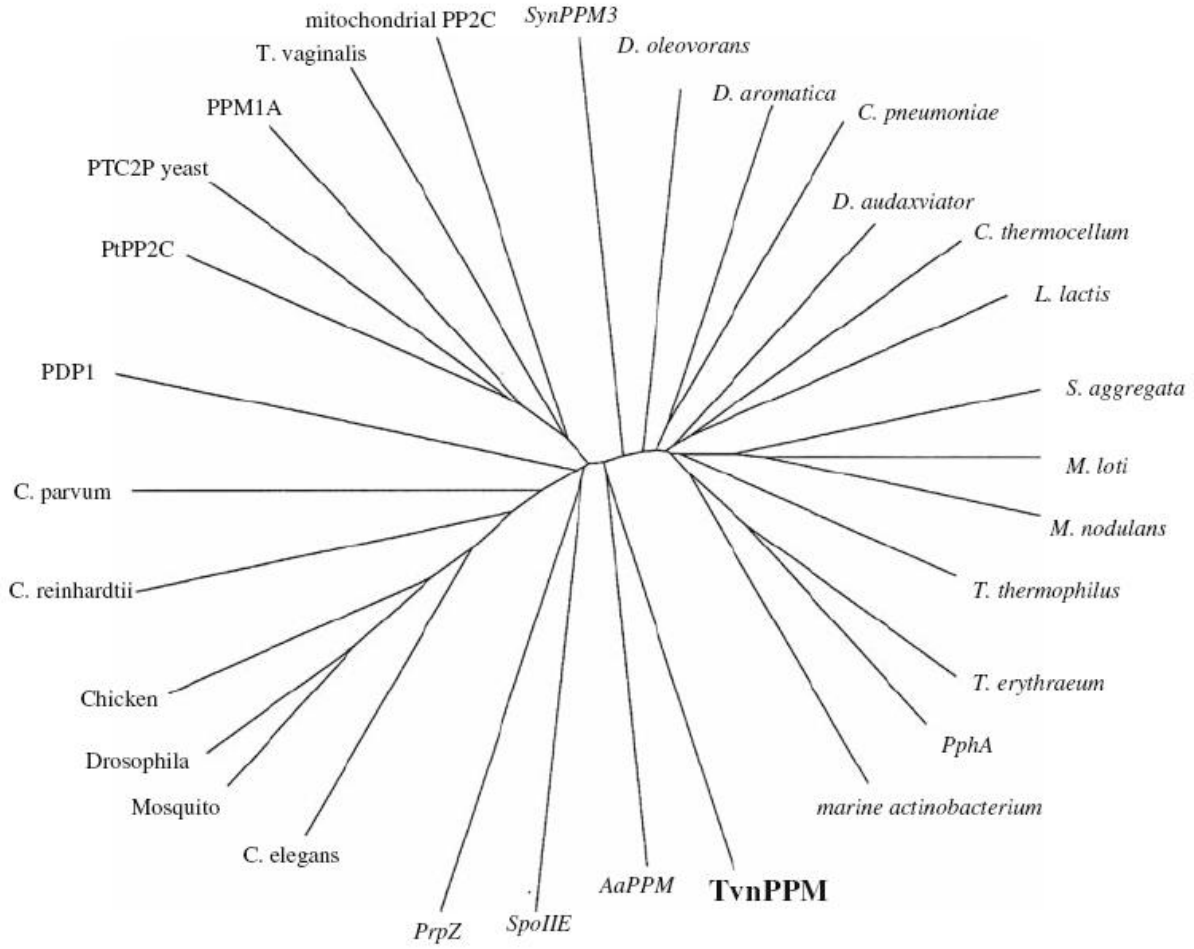

Figure 8-3. Phylogenetic analysis of TvnPPM. Shown is an unrooted tree generated using CLUSTALW available through the Kyoto University Bioinformatics Center (http://align.genome.jp/). TvnPPM is located near the 5:30 position in large bold font. The eucaryal members of the PPM family of protein phosphatases represented include mitochondrial PP2C, a mitochondrial PP2C from *Homo sapiens* (AAX77016); *T. vaginalis*, a putative PPM from the protozoan *Trichomonas vaginalis* G3 (XP_001324774); PPM1A, PPM1A isoform 2 from *H. sapiens* (NP_808820); PTC2P yeast, protein phosphatase type IIC from *Saccharomyces cerevisiae* (AAB64644); PtPP2C, a PP2C protein phosphatase from *Paramecium tetraraurelia* (CAA85448); PDP1, catalytic subunit of pyruvate dehydrogenase phosphatase from cow (P35816); *C. parvum*, a deduced PPM from *Cryptosporidium parvum* (XP_628612); *C. reinhardtii*, a deduced type 2C protein serine/threonine phosphatase from *Chlamydomonas reinhardtii* (XP_001696623); Chicken, TA-PP2C from *Gallus gallus* (XP_415161); Drosophila, a deduced PPM from fruit fly *Drosophila pseudoobscura* (XP_001354256); Mosquito, a putative PP2C from *Aedes aegypti* (XP_001652369); and *C. elegans*, a putative PPM from nematode *Caenorhabditis elegans* (NP_499362). The bacterial members of the PPM family of protein phosphatases represented include PrpZ, the PrpZ protein phosphatase from *Salmonella enterica serovar Typhi* (NP_458902); SpoIIE, the SpoIIE protein phosphatase from *Bacillus subtilis*

(AAB58073); AaPPM, the AaPPM protein phosphatase from *Aquifex aeolicus* (NP_213403); marine actinobacterium, a deduced PPM from marine actinobacterium PHSC20C1 (ZP_01129185); PphA, a PPM from cyanobacterium *Synechocystis* sp. PCC 6803 (NP_440991); *T. erythraeum* protein-serine/threonine phosphatase from *Trichodesmium erythraeum* (YP_722878); *T. thermophilus*, protein phosphatase 2C from *Thermus thermophilus* (YP_005768); *M. nodulans*, a deduced PPM phosphatase from *Methylobacterium nodulans* (ZP_02116970); *M. loti*, a probable PPM protein phosphatase from *Mesorhizobium loti* (NP_103727); *S. aggregata*, a probable PPM protein phosphatase from *Stappia aggregata* (ZP_01546339); *L. lactis*, deduced PPM protein phosphatase from *Lactococcus lactis* (YP_811673); *C. thermocellum*, PPM protein phosphatase from *Clostridium thermocellum* (YP_001037002); *D. audaxviator*, deduced PPM from *Candidatus Desulforudis audaxviator* (YP_001717723); *C. pneumoniae*, deduced PPM from *Chlamydophila pneumonia* (NP_224597); *D. aromatica*, PP2C-like protein from *Dechloromonas aromatica* (YP_283664); *D. oleovorans*, PPM from *Desulfococcus oleovorans* (YP_001529247); and SynPPM3, SynPPM3 protein phosphatase from *Synechocystis* sp. strain PCC 6803 (NP_440091). GenBank accession numbers are given in parentheses

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