The “atypical” protein kinase, SsoPK5, an archaeal member of the piD261/Bud32 subfamily

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ABSTRACT

Open reading frame (ORF) *sso0433* from the archaeon *Sulfolobus solfataricus* encodes a protein kinase, SsoPK5 that exhibits 33% sequence identity to p53 related protein kinase (PRPK) from *Homo sapiens* and 26% sequence identity to piD261/Bud32 from *Saccharomyces cerevisiae*. Given this high degree of similarity, the objectives of this thesis were to (a) clone and purify recombinant SsoPK5, (b) examine its commonalities and differences with its eukaryotic homologues, and (c) determine if it was regulated by nucleotides or related compounds. Substantial progress was achieved on each objective.

After successful cloning of ORF *sso0433* and purification of its protein product, SsoPK5, it was determined that SsoPK5 was cold labile and incubation at 4ºC for an extended period of time rendered SsoPK5 incapable of phosphotransferase activity. When stored at room temperature, SsoPK5 was capable of transferring the γ-phosphate from ATP to casein, reduced carboxyamidomethylated and maleylated (RCM) lysozyme, and p53. SsoPK5 phosphotransferase activity required a divalent metal cofactor; like pid261/Bud32, SsoPK5 preferred Mn^{2+} over the more commonly preferred Mg^{2+}. SsoPK5 was shown to phosphorylate itself on threonine and serine residues; one of the specific amino acid residues modified is threonine-151.

Recombinant SsoPK5 is activated by ADP-ribose and 5'-AMP. Activation was observed when SsoPK5 was stabilized by ATP or a nonhydrolytic analogue, such as β,γ-methylene adenosine 5'-triphosphate (AMP-PCP). Activation was not a result of phosphoryl transfer nor hydrolytic breakdown of ATP or 5'-AMP. This was deduced by the lack of ^{32}P radioactivity incorporated into SsoPK5 during pre-incubation with [γ-^{32}P] ATP for 60 min at 65ºC, and activation by adenosine 5'-O-thiomonophosphate (AMPS), a hydrolysis-resistant analog of AMP. These results may indicate that ADP-ribose acts as a pseudochaperone for SsoPK5 thereby facilitating maximal activity.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ADPR	ADP-ribose
Amp	ampicillin
AMP	adenosine monophosphate
AMPS	adenosine 5'-O-thiomonophosphate
AMP-PCP	β,γ-methylene adenosine 5'-triphosphate
AMP-PNP	adenylyl-imidodiphosphate
ATP	adenosine triphosphate
Bis-tris	2-[Bis (2-hydroxyethyl) imino]-2-(hydroxymethyl)-1, 3-propanediol
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
cAMP	3', 5'-cyclic adenosine monophosphate
CAPS	3-(cyclohenylamion)-1-propane sulfonic acid
CoA	coenzyme A
CPM	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleoside triphosphate
Da	Dalton
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenedioxydiethylenedinitrolo tetraacetic acid
ePK	eukaryotic-like protein kinase
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
G	acceleration of gravity
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kDa	kilodalton
LB	Luria broth
M_r	relative molecular mass
MBP	myelin basic protein
MES	2-(N-morpholino) ethane sulfonic acid
MOPS	3-(N-morpholino) propane sulfonic acid
$NaPP_i$	sodium pyrophosphate
NAD	nicatinamide adenine dinucleotide (oxidized)
NADH	nicatinamide adenine dinucleotide (reduced)
NADP	nicatinamide adenine dinucleotide phosphate (oxidized)
NADHP	nicatinamide adenine dinucleotide phosphate (reduced)

ORF	open reading frame
PAA	phosphoamino acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
PVDF	polyvinylidene fluoride
RCM	reduced carboxyamidomethylated and maleylated
SDS	sodium dodecyl sulfate
TAE	Tris-acetate/EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline, Tween 20
TCA	trichloroacetic acid
TE	Tris EDTA pH 8.0
TLC	thin layer chromatography
Tris	tris(hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

Protein phosphorylation-dephosphorylation: a preeminent regulatory mechanism

Every day, living organisms from bacteria to humans survive in a plethora of surroundings. This survival is dependent on the ability to adapt to changes that may occur in their environment and in turn expose the cell to unknowns. Consequently, organisms must sense and respond to external and internal cues. Cells process these cues via mechanisms collectively known as cellular signal transduction. Cellular signaling is the direct responsibility of cellular proteins. Signaling proteins translate the cues into an output signal by a variety of mechanisms.

One mechanism for processing cellular responses is protein phosphorylation and dephosphorylation. Phosphorylation is a powerful and versatile covalent modification. The phosphate group, with its negative charge and hydrophilicity, is a potent perturber of protein structure (Westheimer 1987; Eichler and Adams 2005). In turn, the removal of a phosphate group also affects the protein's structure. Because of its potency and reversibility, phosphorylation constitutes an ideal process for transmitting cellular information. In fact, it is the most frequent and general device by which biological functions are regulated (Marks 1996). Processes regulated by phosphorylation in eukaryotic organisms include glycogen metabolism (Ramachandran, Angelos et al. 1983), cell cycle progression (Dash and El-Deiry 2005), cellular differentiation (Nakamura, Arai et al. 1998), and neurotransmission (Swope, Moss et al. 1992).

Inappropriate phosphorylation events have been associated with diseases such as cancer (Johnson and Attardi 2006) and diabetes (Sesti 2006).

Protein kinases and phosphatases

Protein kinases catalyze the addition of the γ- phosphate of a nucleotide triphosphate, typically ATP, to a variety of amino acid side chains on acceptor proteins. The acceptor amino acids form a specific type of covalent linkage: phosphomonoester—serine, threonine, tyrosine; mixed acid anhydride—aspartic and glutamic acid; and phosphoramides—histidine, lysine, arginine.

The catalytic domains of eukaryotic-like protein kinases are generally 250-300 amino acids. They possess twelve conserved subdomains (Table 1-1) which serve as characteristic sequence signatures that enable researchers to identify putative protein kinases (Taylor, Knighton et al. 1992). "Typical" eukaryotic protein kinases contain all twelve signature subdomains; however, some eukaryotic protein kinases deviate from this pattern. These protein kinases are referred to as "atypical" eukaryotic protein kinases.

Interestingly, phosphorylation is considered a "reversible" covalent modification. Another set of signaling enzymes—the protein phosphatases—catalyze the hydrolytic removal of phosphate from the amino acid side chain of phosphorylated proteins. The release of phosphate returns the phosphorylated protein to its unmodified state.

Subdomain	Consensus	Function
I	ogxG$_{52}$xogxv	Nucleotide Binding
II	oaoK$_{72}$xo	Nucleotide Binding
III	E$_{91}$xxoo	Nucleotide Binding
IV	h$_{100}$xxooxoxxxo	Structural
V	oooo*oo$_{123}$	Structural
VIa	+oxooh$_{158}$	Structural
VIb	oohrD$_{166}$ok+xNooo	Phosphotransfer Catalysis
VII	oko+D$_{184}$fgo+	Metal-Ion Cofactor Interaction
VIII	+pE$_{208}$oo	Protein Substrate Binding
IX	D$_{220}$oo+ogooo	Salt Bridge Formation
X	ooxxo$_{250}$	Structural
XI	R$_{280}$x+	Salt Bridge Formation

Table 1-1. ePK superfamily subdomains

Sequence numbering is based on mouse cAMP-dependent protein kinase Cα. Uppercase letters: universally conserved residues; lowercase letters: highly conserved residues; 'o': non-polar residues; *: polar residues; 'x': any amino acid; +: small residues with near neutral polarity. Highlighted regions represent the most critical subdomains conserved amongst even atypical ePKs.

Adapted from Hanks and Hunter (1995).

Protein phosphorylation-dephosphorylation in the Bacteria and the Archaea

For many years, serine, threonine, and tyrosine protein kinases were thought to be an exclusively eukaryotic phenomenon that had evolved to manage the complexities of multicellular organisms (Kennelly and Potts 1996). However, in 1991, a serine/threonine protein kinase, *pkn1*, was discovered in *Myxococcus xanthus*. Pkn1 shared 27% - 31% sequence identity with several protein kinases from eukaryotes, such as the cAMP dependent protein kinase and protein kinase C (Munoz-Dorado, Inouye et al. 1991). Subsequent research has shown that serine- and threonine-phosphorylation modulates several processes in bacteria including various aspects of metabolism (Deutscher, Francke et al. 2006), cell division (Jin and Pancholi 2006), and nitrogen fixation (Monson, Ditta et al. 1995). With the discovery of eukaryotic-like protein kinases (ePK) in bacteria, researchers began to evaluate the type(s) of protein kinases resident in the third phylogenetic domain, the *Archaea*. In 1980, Spudich and coworkers were the first to report the presence of phosphorylated proteins and protein kinase activity in the *Archaea* (Spudich and Stoeckenius 1980). In 1984, Skórko detected the presence of both phosphorylated proteins and protein kinase activity in *Sulfolobus acidocaldarius*. Skórko noted that the degree of phosphorylation was dependent on the stage of growth being observed. Moreover, he observed that phosphorylation occurred on serine and threonine amino acid residues (Skorko 1984). In 1999, Bischoff and Kennelly observed phosphotransferase activity associated with a 67kDa protein from *S. solfataricus* (Bischoff and Kennelly 1999). Utilizing conserved motifs, many potential eukaryotic-

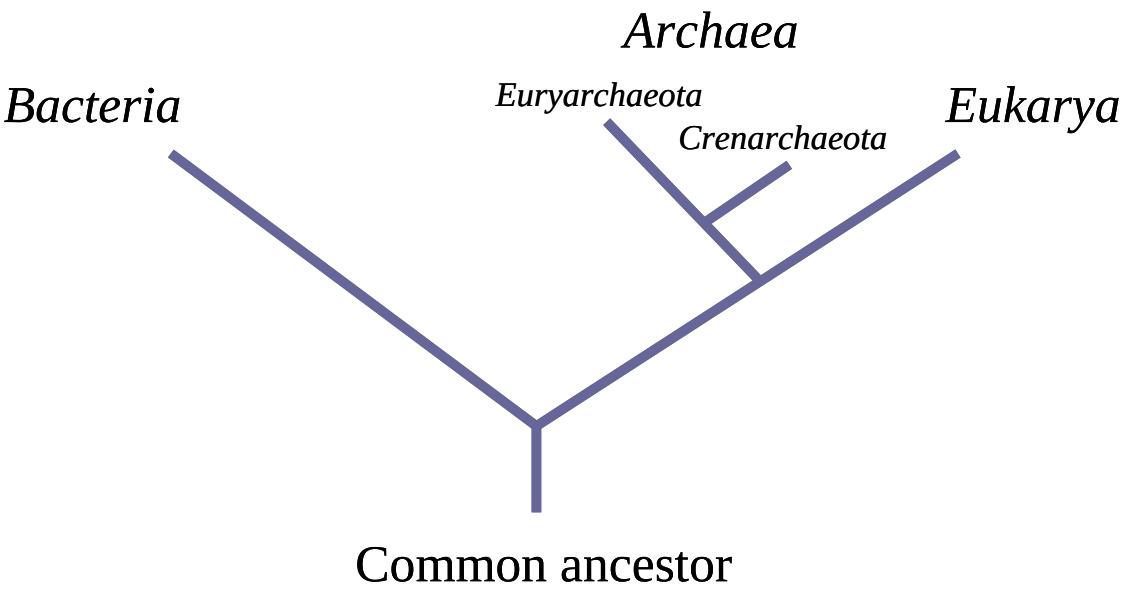

Figure 1-1. The "Tree of Life"

The illustration above depicts *Archaea* as sharing a common ancestor with *Eukarya* more recently than *Eukarya* and *Bacteria*. The *Euryarchaeota* and the *Crenarchaeota* are the two major subdivisions of the archaea.

like protein kinases have been annotated in both the *Bacteria* and *Archaea* (Shi, Potts et al. 1998; Kennelly 2003). In the *Archaea,* these include a very high proportion of "atypical" eukaryotic protein kinases.

The three domains of life

Decades after it was originally proposed (Woese and Fox 1977), the archaeal hypothesis still provides the best explanation for the vast amount of molecular and biochemical diversity of contemporary prokaryotic organisms (Whitman, Pfeifer et al. 1999). The hypothesis states that formerly prokaryotic organisms are distributed between two very ancient phylogenetic lineages (Woese and Fox 1977). The former classification of living organisms into the Prokaryotes and the Eukaryotes was superseded by the current classifications into three domains: the *Eukarya*, the *Bacteria*, and the *Archaea* (Woese and Fox 1977; Woese, Kandler et al. 1990). Using the three domain organization, the designations "eukaryote" and "prokaryote" represent a morphological description rather than a phylogenetic one. Eukaryotic organisms possess an internal nuclear membrane, while prokaryotic organisms do not. Morphologically archeaotes are prokaryotes; however, when nucleotides sequences, particularly rRNA sequences, are compared, they do not resemble eubacteria any more, perhaps less, than they resemble eukaryotes (Woese, Kandler et al. 1990). The most universally accepted "Tree of Life" is depicted in Figure 1-1; it portrays a progressive emergence of the three domains (Doolittle 1995).

In 1990, the *Archaea* were subdivided into two major groups based on rRNA sequences: the *Crenarchaeota* and the *Euryarchaeota* (Figure 1-1). The euryarchaeotes

are a diverse phylogenetic group, encompassing a range of phenotypes: methanogens, extreme halophiles, and sulfate-reducing species. The crenarchaeotes form a more phylogenetically homogenous group, comprising of all thermophilic archaeons (Woese, Kandler et al. 1990). Since then, many more crenarchaeotes have been discovered, including several mesophiles. In addition, two additional subdivisions have been proposed: the *Koracheota* and the *Nanoarchaeota*. These subdivisions arose from the rRNA differences observed between some of the recently discovered archaeal species in the Icelandic hot springs. More data are necessary to ensure that these prokaryotes require a unique archaeal subdivision (Cracraft and Donoghue 2004).

Characteristics of the *Archaea*

The *Archaea* share a number of characteristics with the *Bacteria*: similar genome size, high density of coding sequences in their genome, the presence of polycistronic RNA, and the lack of a nuclear membrane. Like many of the *Bacteria*, archaeal cell walls are constructed of a polysaccharide similar to peptidoglycan consisting of alternating N-acetylglucosamine and N-acetyltalosaminuronic acid repeats, polysaccharides, glycoproteins or proteins. In *Archaea*, the cell wall is known as pseudopeptidoglycan. Halophiles, organisms that survive in environments with high concentrations of salt, contain negatively charged amino acids in their cell walls, which act to neutralize the positive charges of the high Na^+ environment, to prevent cell lysis (Madigan and Marrs 1997).

On the other hand, the sequences of many archaeal proteins exhibit greater similarity to the corresponding proteins in the *Eukarya* than in the *Bacteria*. For

example, the DNA replication machinery of the *Archaea* bears a striking resemblance to that of the *Eukarya*. Multiple origins of replication have been reported in some Archaeal genomes. In *Sulfolobus solfataricus,* all three identified origins of replication are utilized throughout the cell cycle (Lundgren, Andersson et al. 2004). DNA-binding proteins, homologous to eukaryal histones, have been identified in many euryarchaeons, although only one homologue to date is known in the *Crenarchaeota* (Sandman and Reeve 2006). However, crenarchaeotes such as the Sulfolobales do possess DNA proteins that are functionally similar to histones such as Alba, Alba2, and Sul7d (White and Bell 2002; Jelinska, Conroy et al. 2005). Archaeons and eukaryotes share other proteins involved in DNA replication: helicases, single-stranded binding DNA proteins, primases, sliding clamps, and DNA polymerases, some of which possess 3'-5' exonuclease activity (Barry and Bell 2006).

Archaeal-eukaryal similarities extend to transcription and translation as well. Sequence comparisons suggest that archaeal RNA polymerases are more similar to eukaryal RNA polymerases than to bacterial ones (Werner 2007). Both archaeal and eukaryal RNA polymerases require the assistance of a similar set of transcriptional regulatory proteins in order to be recruited to their cognate promoter sequences: initiation factors, TATA-box-binding protein, and transcription factor B (Bell and Jackson 2001). The genes encoding archaeal 23S rRNA and 16S rRNA, as well as many tRNAs, contain introns (Dalgaard and Garrett 1992; Armbruster and Daniels 1997).

In contrast to eukaryotes, archaeal mRNAs are not polyadenylated, may be polycistronic, and often contain the Shine-Dalgarno motif (Madigan and Marrs 1997). Moreover, the sequences of the proteins that comprise archaeal ribosomes have

significant similarity to their eukaryal counterparts (Smith, Lee et al. 2008). While the *Archaea* have 70S ribosomes, translation is not inhibited by tetracycline, erythromycin, chloramphenicol, streptomycin, and kanamycin as in bacteria. It therefore appears that the translation machinery of the *Archaea* employs a mechanism more homologous to that found in the *Eukarya* (Madigan and Marrs 1997). The *Archaea*, like the *Eukarya*, use methionyl tRNA, instead of N-formyl-methionyl tRNA, to initiate translation (Lee, Choi et al. 1999).

The *Archaea* display a number of unique characteristics as well. For example archaeal membranes are either bilayers or monolayers constructed of chemically unique lipids, predominantly consisting of glycerol diethers and glycerol tetraethers, respectively (Sprott 1992; Cracraft and Donoghue 2004). Bacterial and eukaryal membranes are bilayers of lipids predominantly consisting of fatty acid side chains linked to glycerol via ester linkages. By contrast, archaeal lipids consist of a glycerol molecule bonded to polyisoprene molecules by ether linkages (Madigan and Marrs 1997).

Why study protein kinases in *Sulfolobus solfataricus*?

Given that contemporary phylogenetic trees group the archaeal and eukaryotic domains on a common branch (Figure 1-1), the *Archaea* appeared to offer the greater long-term promise of the two prokaryotic domains in a search for protein kinases and protein phosphatases that exhibited recognizable homology in sequence and possibly even biological function to those in eukaryotes. Among the prokaryotes, *S. solfataricus* may represent one of the most similar extant species to eukaryotic organisms. It is thus possible that this archaeon would enable researchers to study protein phosphorylation

events pertaining to medically relevant organisms on a smaller scale and more tractable scale. The human genome is predicted to encode more than 500 protein kinases (Manning, Whyte et al. 2002). From 30% to 50% of eukaryotic proteins are predicted to undergo phosphorylation (Cohen 2002). The sheer quantitative complexity of eukaryotic phosphorylation-dephosphorylation networks suggests that the development of smaller models would offer important insights into their operating principles. Analysis of several archaeal genomes indicate that members of the *Archaea* contain approximately 100-fold fewer protein eukaryotic-like protein kinases than the typical mammal (Kennelly 1998).

Why, from amongst the *Archaea*, was, *Sulfolobus solfataricus* selected as our "model organism?" First, the *S. solfataricus* genome was sequenced in 2001 (She, Confalonieri et al. 2000; She, Singh et al. 2001). Second, it is relatively easy to grow in a laboratory. It is an aerobe with an optimum pH of 1.0 - 3.0 and growth temperature of 60^{o} - 90^{o}C (Lubben and Schafer 1989). Third and perhaps more importantly, *S. solfataricus* is considered among the most 'eukaryotic-like' members of the *Archaea* (Woese and Wolfe 1985). In addition, a genetic system for *S. solfataricus* is currently in development. Recently, several loss of expression mutants have been constructed (Schelert, Dixit et al. 2004). While *S. solfataricus* may appear to be a simplistic organism, in actuality it represents a remarkably complex one. It exhibits a broad range of metabolic activities that have enabled it to inhabit a geographically diverse range of environments characterized by their extreme temperatures and acidity (Madigan and Marrs 1997). It also possesses several likely sensor response proteins (Klenk, Clayton et al. 1997), including one of the largest sets of potential eukaryotic protein kinases [see

below], that should provide valuable insight into archaeal molecular regulatory mechanisms.

Protein phosphorylation in *S. solfataricus*

The *Archaea* encode a variety of known cellular signaling proteins: protein serine/threonine/tyrosine kinases, protein—serine/threonine/tyrosine—phosphatases, protein acetylases and deacetylases, and adenylate cyclases. Differences in the levels of protein phosphorylation have been observed in several of the *Archaea*, including *S. solfataricus*, following environmental changes (Eichler and Adams 2005). Protein phosphorylation has been implicated in the control of archaeal DNA replication and the cell cycle (Bell 2002). Based upon both phylogenetic and physiological analysis, it would appear that protein phosphorylation is one of the more ancient means of cellular signaling.

The typical ePK subfamily dominates the population of protein kinases encoded by eukaryotic genomes. This protein kinase family also predominates among the eukaryote-like protein kinases in the *Bacteria*. (These ePKs were acquired via lateral gene transfer from the *Eukarya.*) (Doolittle 1999) Eukaryotic organisms also contain a handful of ePKs that deviate from the typical ePK paradigm. These are referred to as atypical eukaryote-like protein kinases. Atypical protein kinases generally lack the lysine or arginine residue conserved within the catalytic loop, also called subdomain VIb (Table 1-1). Unlike bacterial and eukaryotic organisms, archaeons are rich in atypical eukaryotic-like protein kinases. For example, the genome of *S. solfataricus* is predicted to encode as many as eleven putative eukaryote-like protein kinases, only three of which are potential typical ePKs (Table 1-2).

Open Reading Frame (ORF)	Class of protein kinase
sso2291	Typical ePK
sso3207	Typical ePK
sso3182	Typical ePK
sso0433	**piD261/PRPK**
sso2374	RIO protein kinase
sso0197	RIO protein kinase
sso2605	ABC1 family
sso1038	AQ578 family
sso0361	AQ578 family
sso2387	"other" ePKs
sso0469	"other" ePKs

Table 1-2. Putative eukaryotic-like protein kinases in *S. solfataricus*.

Protein kinases in *S. solfataricus* can be grouped into the typical and five atypical families. The polypeptide encoded by ORF *sso0433* is the protein kinase of interest in this study.

piD261/Bud32 family of protein kinases

The piD261/Bud32 subfamily of atypical protein kinases were named for the *Saccharomyces cerevisiae* protein kinase encoded by the *YGR262c* gene. ΔYGR262c mutants exhibit a severe growth phenotype, reduced survival during S phase of cell division, and random budding (Sartori, Mazzotta et al. 2000). Homozygous diploids fail to enter sporulation and display no visible evidence of meiotic division (Briza, Bogengruber et al. 2002). Moreover, these mutants show alterations in cell wall structure when compared to wild type yeast (Piet W. J. de Groot 2001). The phosphotransferase activity of piD261/Bud32 is activated by the phosphorylation of Ser-187 and/or Ser-189 (Facchin, Lopreiato et al. 2003). A broad range of proteins has been postulated to interact with piD261/Bud32. These include, but are not limited to, ATPases, cell division cycle proteins, and glutaredoxin (Ho, Gruhler et al. 2002). Additional research has shown that the pid261/Bud32 protein kinase, along with Kae1p (a putative endopeptidase), are components of the kinase, putative endopeptidase, and other proteins of small size (KEOPS), also known as the endopeptidase-like kinase chromatin-associated (EKC) complex. The KEOPS/EKC complex has been shown to regulate telomerase, specifically in telomere capping and elongation (Downey, Houlsworth et al. 2006). The EKC complex has been reported to be involved in transcription (Kisseleva-Romanova, Lopreiato et al. 2006). In both cases, piD261/Bud32 is an essential member of the complex; however, it is unknown what specific role piD261/Bud32 plays in these complexes (Downey, Houlsworth et al. 2006; Kisseleva-Romanova, Lopreiato et al. 2006).

Homo sapiens possess a homolog of a piD261/Bud32, known as p53 related protein kinase (PRPK). p53 regulates the fate of a cell after DNA damage. Its gene ranks amongst the most commonly mutated in cancerous tumors (Franks and Teich 1997). PRPK phosphorylates p53 on Ser15 *in vitro*; this phosphorylation event has been shown to activate p53. Intriguingly, activation of PRPK *in vitro* required pre-treatment with a lysate from COS-7 fibroblast cells, the cell line in which the recombinant protein had been over-expressed. Subcellular localization studies indicate PRPK is found in the nucleus (Abe, Matsumoto et al. 2001). Interestingly, PRPK partially complements the ΔYGR262c mutant, indicating that these protein kinases may share a significant degree of functional conservation. When PRPK is over-expressed in mutant yeasts, the slow growth phenotype is partially rescued. Additionally, piD261/Bud32 has been shown to phosphorylate p53 on Ser15 and Ser37 *in vitro* (Facchin, Lopreiato et al. 2003).

The piD261/Bud32 subfamily protein kinases contain a shorter catalytic domain than typical ePKs and lack the conserved lysine residue in subdomain VIb as well as all of subdomain XI (Facchin, Lopreiato et al. 2002). The members of the piD261/Bud32 protein kinase prefer to phosphorylate serine/threonine residues surrounded by acidic residues as opposed to the basic residues typically encountered at most other sites of serine/threonine phosphorylation. The piD261/Bud32 protein kinases therefore are sometimes referred to as acidophilic protein kinases (Facchin, Sarno et al. 2002). Recently, another homologue of piD261/Bud32 and PRPK has been reported in *Pyrococcus abyssi* (Hecker, Graille et al. 2009) and *Methonacoladococcus jannaschii* (Hecker, Lopreiato et al. 2008; Mao, Neculai et al. 2008). It has been reported in *P. abyssi* that Kae1 inhibits the autophosphorylation activity of the piD261/Bud32

homologues from both *P. abyssi* and *M. jannaschii* (Hecker, Lopreiato et al. 2008; Hecker, Graille et al. 2009).

Specific aims and significance

Protein kinases transfer the γ-phosphate of ATP (or GTP) to acceptor proteins. Typical eukaryotic-like protein kinases (ePK) are defined by a catalytic domain of 250-300 amino acids containing twelve (I, II, III, IV, V, VIa, VIb, VII, VIII, IX, X, XI) conserved subdomains. Three roles are attributed to the catalytic domains: the binding and orientation of ATP or GTP as a complex with a divalent metal, the binding and orientation of the protein substrate, and the transfer of the γ-phosphate from the nucleotide to the acceptor protein (*The Protein Kinase Facts Book* 1995).

The piD261/Bud32 subfamily differs in several respects from the majority of eukaryotic protein kinases and therefore is often referred to as an atypical subfamily of eukaryotic-like protein kinase. piD261/Bud32 protein kinases are considerably shorter in length than their typical counterparts. In fact, in 1997 piD261/Bud32 from *Saccharomyces cerevisiae* was the smallest known protein kinase (Stocchetto, Marin et al. 1997). Other characteristics that differ from typical protein kinases are the lack of conserved basic residues in the subdomain VIb and the acidophilic nature of the phosphorylation sites on the acceptor proteins (Facchin, Sarno et al. 2002). The piD261/Bud32 subfamily is particularly unique because every archaeon and eukaryote possesses a potential homologue. As it is the only ePK that can make this claim, the piD261/Bud32 protein kinases may be the most ancient protein kinases extant (Facchin, Lopreiato et al. 2003).

Open reading frame (ORF) *sso0433* from the archaeon *Sulfolobus solfataricus* encodes a protein kinase, designated as SsoPK5 that exhibits 33% sequence identity to PRPK and 26% sequence identity to piD261/Bud32. Given this high degree of similarity, we hypothesize that functional conservation exists between SsoPK5 and the previously-characterized, but functionally cryptic, members of the piD261/Bud32 subfamily. Its smaller genome and smaller kinome render *S. solfataricus* a seemingly more reasonable organism for elucidating a physiological role of the piD261/Bud32 family. As the first step in this process, a plan of investigation was developed around the following aims:

1) **What characteristics does SsoPK5 share with piD261/Bud32 and PRPK?**

    a. How does the SsoPK5 primary sequence compare to its homologues in eukaryotes?
    b. What are the characteristics of the sites it phosphorylates?
    c. What specific characteristics do SsoPK5 and its eukaryotic counterparts have in common?
    d. Can SsoPK5 phosphorylate p53 *in vitro*?

2) **Is the activity of SsoPK5 affected by nucleotides and related metabolites endogenous to *S. solfataricus*?**

    a. What types of nucleotides and nucleotide derivatives affect the activity of SsoPK5?
    b. What is the nature of their effect(s) on the activity of SsoPK5?
    c. Do these nucleotides and nucleotide derivatives act at physiologically relevant concentrations?

3) **Is autophosphorylation necessary for protein kinase activity?**

    a. Do the activity levels decrease when threonine-151 is converted to Ala or Asp?
    b. Can we artificially increase the activity of SsoPK5 by altering subdomain VIb?

CHAPTER II

MATERIALS AND METHODS

Materials

[γ-^{32}P] ATP was purchased from Perkin Elmer (Waltham, MA). Chelating Sepharose Fast-Flow was from Amersham Biosciences (Piscataway, NJ). Pre-stained standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Richmond, CA). *NcoI*, *SalI*, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Protease inhibitor cocktail minus EDTA was from Roche Applied Science (Indianapolis, IN). *Pfu Turbo* DNA polymerase, *Pfu Ultra* DNA polymerase, *DpnI*, BL21-CodonPlus (DE3)-RIL cells, and Quik-Change II site-directed mutagenesis kits were from Stratagene (LaJolla, CA). Coomassie protein assay reagent and bovine serum albumin (BSA) were purchased from Pierce Biotechnology (Rockford, IL). QIAquick PCR purification and QIAprep spin miniprep kits were from QIAGEN (Valencia, CA). 1 kb DNA ladder and 5X DNA loading dye were purchased from Bio-Rad (Hercules, CA). Expression vector pET29b and pET21d were from Novagen (San Diego, CA). *Escherichia coli* α-select cells were from Bioline (Taunton, MA). Custom oligonucleotides were purchased from Sigma Genosys (St. Louis, MO). DNA sequencing reactions were processed at the Virginia Tech Bioinformatics Institute Core laboratory DNA sequencing facility (Blacksburg, VA). Genomic DNA from *Sulfolobus solfataricus* P2 was from the American Type Culture Collection (ATCC) (Manassas, VA). The p53-GST fusion protein was purchased from Santa Cruz Biotechnology (Santa

Cruz, CA). Immobolin-P PVDF membranes were purchased from Millipore (Bedford, MA). Anti-phospho-p53 antibodies were from Cell Signaling Technologies (Danvers, MA). SDS-PAGE prestained standards, SDS page electrophoresis apparatus, and electroblotting transfer apparatus were purchased from Bio-Rad (Hercules, CA). General laboratory reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Microfuge tubes and pipet tips were from Phenix Laboratory Supplies (Candler, NC).

Molecular biology

Luria broth (LB), LB plates, Tris-EDTA pH 8.0 (TE), 0.5 M EDTA, and stock solutions of ampicillin, chloramphenicol, kanamycin, IPTG and 6X gel-loading buffer were prepared as described in *Molecular Cloning, A Laboratory Manual* (Sambrook, Maniatis et al. 1989). For daily use, *Escherichia coli* (*E. coli*) were streaked on LB agar plates containing the appropriate antibiotic and grown on LB media supplemented with the appropriate antibiotic. For long-term storage, an 800 µl aliquot of an overnight culture of *E. coli* was mixed with 200 µl sterile 80% (w/v) glycerol and stored at -80°C.

Agarose gel electrophoresis

Samples of genomic or plasmid DNA were routinely resolved into their individual oligonucleotide components using agarose gel electrophoresis (Sambrook, Maniatis et al. 1989). Gels contained 0.8% - 1.0% (w/v) agarose depending upon the size range of DNA to be separated, in 0.04 M Tris-acetate, 1mM EDTA (TAE) buffer pH 8.0 containing

0.4% (w/v) ethidium bromide (EtBr). DNA samples were prepared by mixing 5 volumes of the sample with one volume of Bio-Rad 5X loading buffer. Electrophoresis was conducted at 100 volts in TAE buffer for approximately 45 min.

Cloning of ORF *sso0433*

ORF *sso0433* was amplified by polymerase chain reaction (PCR) in a volume of 50µl containing 550 ng of *S. solfataricus* genomic DNA as template, 5 pmol each of the forward and reverse primers, 200 µM each dATP, dCTP, dGTP, and dTTP, 5 µl 10x reaction buffer (Stratagene), and 1 unit *Pfu* Turbo DNA polymerase. The sequences of the forward and reverse primers were, respectively, 5'GATATTCCATGGAGAAATTAAGGTTAATTAAGCGTGGC GC-3' and 5'-TTATTTGTCGACTTCGTTAATACTCCTATTTTTACGCCTC-3'. The thermocycler was programmed for an initial 2.5 min incubation period at 94°C to denature the genomic DNA. This was followed by thirty cycles of denaturing at 92°C for 15 s, annealing at 45°C for 30 s, and elongation at 72°C for 30 s. A final elongation step at 72°C for 7 min was done to ensure that all PCR products were full length and 3'-adenylated. The resulting ~670 bp PCR product was cloned into vector pET29b as follows. 20 µl of the PCR product was incubated with the restriction enzymes *NcoI* and *SalI*, 1 unit each, at 37ºC for 4 to 16 hours. The pET29b vector was also incubated with the restriction enzymes *NcoI* and *SalI*, 1 unit each, at 37ºC for 4 to 16 hours. The digested PCR product was ligated into the digested pET29b vector, which added oligonucleotides encoding an N-terminal S-tag and a C-terminal 6x-His-tag using T4 DNA ligase. The resulting plasmid was used to transform *E. coli* strain α-select from Bioline. The transformed cells

were cultured overnight on LB plates containing 50 μg/ml kanamycin. The plasmid was isolated from LB media containing *E. coli* cells harboring pET29b using the QiaPREP Miniprep Kit according to manufacturer's protocol. DNA sequence analysis of the cloned DNA insert was performed at the Virginia Tech DNA Sequencing Facility to verify the fidelity of PCR amplification.

A similar procedure was followed for the pET21d, which codes for a C-terminal 6x-His-Tag fused onto SsoPK5. The same restriction enzymes were utilized. Since pET21d codes for ampicillin as opposed to kanamycin resistance; the LB plates and media both contained 100 μg/ml ampicillin.

Site-directed mutagenesis

Site-directed mutagenesis of cloned ORF *sso0433* was performed by using Stratagene's Quik Change Mutagenesis kit following the manufacturer's suggested protocols. Primers were designed to minimize base-pair changes. In addition, *Pfu Ultra*, a high fidelity DNA polymerase from Stratagene, was utilized in all of the site-directed mutagenesis-related PCR reactions. The thermocycler was programmed for an initial incubation period at 95°C for 30 sec to denature the plasmid DNA. This was followed by a series of 16 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 68°C for 1 min. The reaction was cooled to 37ºC or below. The annealing temperature was dependent on the composition and length of the primers. Following PCR, the samples were treated with 1 unit of *DpnI* for 2 hours at 37ºC. After *DpnI* treatment, the samples were transformed into α–select *E. coli* cells. The transformants were incubated in 5 ml LB media containing 50 μg/ml kanamycin (pET29b) or 100 μg/ml

ampicillin (pET21d) at 37ºC for 16 hours. Plasmids were isolated from α–select *E. coli* cells with a QiaPREP Miniprep Kit according to manufacturer's protocol. The plasmids were then subjected to DNA sequencing at the Virginia Tech Bioinformatics Institute core laboratory DNA Sequencing Facility to verify that the desired base changes had been introduced.

DNA sequencing

DNA sequencing was done at the Virginia Tech DNA Sequencing Facility. Plasmids, pET29b or pET21d, used as templates for DNA sequencing were isolated from 5 mL overnight cultures of *E. coli* grown in LB media supplemented with kanamycin 50ug/ml or ampicillin 100ug/ml, respectively. Plasmids were purified using QiaPREP Miniprep Kit according to manufacturer's protocol. For sequencing, the plasmids were resuspended in 60% of the manufacturer's suggested volume of sterile E-pure water. The plasmids were sequenced by utilizing the T7 promoter and T7 terminator sequences encoded by the expression vectors, pET29b or pET21d.

Expression of recombinant protein in *E. coli*

Recombinant proteins were routinely overexpressed in *E. coli* as follows. First, approximately 50 - 100 ng of pET29b or pET21d plasmid containing ORF *sso0433* from *S. solfataricus* suspended in a volume of 1 µl of sterile E-pure water was transferred to a vial of chilled BL21 (DE3) RIL Chemically Component *E. coli* cells purchased from Stratagene. The vial was placed back on ice. Occasionally, the contents of the vial were

divided to accommodate two different constructs. Following a 30 min incubation on ice, the mixture was incubated at 42°C for 30 s without shaking (heat shock). The vial was then returned to the ice. Next, 250 µl of room temperature SOC media was added, the tubes capped tightly, and the contents agitated with the tube in a horizontal position at 200 rpm at 37ºC for 30 min. The entire transformation mixture, containing either a pET29b or pET21d construct, then was added to a 16 x 125 mm snap cap sterile tube containing 5 ml of LB media containing chloramphenicol (34 µg/ml), and either kanamycin (50 µg/ml) or ampicillin (100 µg/ml), respectively. The culture was incubated overnight at 37°C with shaking (225 rpm). The following day, as described previously, glycerol permanents were made from the overnight cultures and stored at -80°C for future use. The remainder of the culture, containing either a pET29b or pET21d construct, was used to inoculate 200 ml of LB media containing chloramphenicol (34 µg/ml), and either kanamycin (50 µg/ml) or ampicillin (100 µg/ml), respectively, in a 1 L flask. The culture was incubated at 37°C with constant shaking at 225 rpm until the OD_{600nm} reached 0.5 - 0.8 (approximately 2 hours). Overexpression of the recombinant protein was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), 200 µl of 1 M stock solution, to a final concentration of 1 mM. After incubating for an additional three hours at 37ºC (induction), the cells were collected by centrifugation at 1000 x g for 15 min at 20ºC. The cell pellet was immediately resuspended for purification, as described below.

Protein purification

Cells from a 200 ml culture (see above) were immediately resuspended in 4 ml of 50 mM Bis-tris, pH 7.0, containing EDTA-free protease inhibitor cocktail. Lysozyme

was to a final concentration of 50 μg/ml and added to the mixture, which then was incubated at room temperature for 15 min. The cells were then lysed on ice by sonic disruption using 4 x 30 sec bursts at 30% intensity of the Heat Systems-Ultrasonics, Inc, Model W185 Sonifier Cell Disruptor, equipped with a microprobe, with 30 s on ice between the bursts. The sonicated mixture was subjected to centrifugation at 3,000 x g for 15 min at 20°C to remove insoluble material, and the supernatant liquid (soluble cell extract) was incubated for 10 min at 60°C. The soluble cell extract was then centrifuged at 3,000 x g for 15 min at 20°C to remove aggregated proteins and other insoluble material. The supernatant liquid was then stored at room temperature. The resulting protein product was designated recombinant SsoPK5, which stands for recombinant S. *solfataricus* Protein Kinase 5.

Protein concentration assays

Protein concentrations were determined by the method of Bradford (Bradford 1976) using Coomassie Protein Assay Reagent and bovine serum albumin (BSA) as standard from Pierce Biotechnology. A standard curve was generated with BSA at a stock concentration of 0.2 mg/ml. An individual sample's protein concentration was obtained by creating several serial dilutions of the sample. The absorbance of the mixtures was measured in duplicate at 595 nm. The mixtures whose mean absorbance fell within the linear range of the curve were used to calculate the protein concentration in the original sample. If more than one mixture's absorbance fell within this range, an average of the measured protein concentrations was used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide resolving gels, 12% (w/v) acrylamide running gels, 5% (w/v) acrylamide stacking gels, and 25mM Tris, 192mM glycine, 0.1% (w/v) SDS (running buffer) were prepared as previously described (Laemmli 1970). Generally, the resolving gels were polymerized in advance and stored for up to 10 days prior to use at 4ºC in plastic wrap. For preparation of protein samples one volume of 4X SDS loading buffer consisting of 200mM Tris pH 6.8, containing 400mM DTT, 8% (w/v) SDS (electrophoresis grade), 0.4% (w/v) bromophenol blue, and 40% (v/v) glycerol was added to four volumes of sample (Sambrook, Maniatis et al. 1989). The resulting mixture was agitated on a vortex mixer until visibly homogeneous, then heated for 5 min at 100°C. The sample was subjected to a brief centrifugation (10,000 x g for 30 s at room temperature) and the mixture loaded into the wells of the SDS-polyacrylamide gel. Bio-Rad pre-stained molecular weight markers were loaded in one well of the gel. These standards were used to estimate the molecular weights of the sample proteins. Gels were run at a constant current of 25 mAmp/gel until all the pre-stained standards entered the running gel, following which the current typically was increased to 50 mAmp/gel. Upon completion, the gels were transferred from the apparatus to approximately 25ml of 10% (v/v) glacial acetic acid containing 45% (v/v) methanol and 0.075% (w/v) Coomassie R-250 Brilliant Blue (staining solution).

Western blotting

Following SDS-PAGE, proteins within the SDS-polyacrylamide gel were sometimes transferred to an Immobilon-P PVDF membrane using a wet transfer

electroblotting system. First, the Immobilon-P PVDF membrane was wetted with 100% Methanol for 30 s, rinsed with e-Pure H_2O for 2 min, and then immersed in blotting buffer (10mM CAPS pH 11.0, containing10% (v/v) methanol) for at least 5 min at room temperature. The SDS-polyacrylamide gel and the Immobilon-P PVDF were placed together in the blotting apparatus according to manufacturer's instructions and then subjected to an electric field of 100 volts (current limit 1 .0 AMP) for 1 hour at 4°C. At the end of this period, the PVDF membrane was removed from the transblotting apparatus, rinsed with methanol, and air dried at room temperature.

Detection of proteins in SDS-polyacrylamide gels

Proteins in SDS-polyacrylamide gels were detected by staining with Coomassie Blue R-250 (Sambrook, Maniatis et al. 1989). After soaking in the staining solution for periods of 2 hours to overnight with gentle agitation the SDS-polyacrylamide gels were destained by soaking a solution of 10% (v/v) glacial acetic acid containing 20% (v/v) methanol (destaining solution), with gentle agitation, until little to no background remained, usually 24-48 hours. During the destaining process, a sponge or some Kim-Wipes were placed in the container to absorb the dye as it diffused out of the SDS-polyacrylamide gel. The gels were removed from the detaining solution and wrapped in plastic wrap. Their visible image was digitally recorded using a Du Pont Instruments Bio-Rad ChemiDoc XRS (170-8070) with Quantity One Software and Universal Hood II (W/TLUM 100/240 Vac). Gels were stored wrapped in plastic at room temperature.

Immunological detection of proteins on membranes

Following Western transfer, once the Immobolin-P PVDF membrane was completely dry, it was incubated with commercial antibodies against the proteins of interest. For example, anti-phos-p53 antibodies were purchased from Cell Signaling Technology for six different known phosphorylation sites on p53. The protocol described below is adapted from a combination of the antibody and membrane manufacturers' instructions. The membrane was incubated with gentle agitation for 1 hour at 25ºC with 10 ml of the primary antibody dilution (1:1000) in Tris-Buffered Saline containing 0.1% (v/v) Tween-20 (TBS-T) containing 1% (w/v) BSA (blocking buffer). Next, the solution containing the primary antibody solution was decanted, saved, and stored at 4ºC. The membrane was rinsed three times with TBS-T. The membrane was then incubated with gentle agitation at 25ºC for 30 min 10 ml of blocking buffer containing a 1:500 dilution of the secondary antibody, anti-rabbit IgG-Horse Radish Peroxidase (HRP)-linked. Areas of the membrane containing high levels of the secondary antibody were visualized by taking advantage of the activity of its horse radish peroxidase reported using Pierce's SuperSignal West Femto Substrate per the manufacturer's protocol.

Solution assay of protein kinase activity

Phosphotranferase activity was assayed in solution using the filter paper method described by Walsh et. al (Walsh, Brostrom et al. 1972). Samples containing SsoPK5 were incubated at 65^{o} C in a volume of 50 µl of 50 mM Bis-tris, pH 7.0, containing 100 µM ATP (300-1000 µCi of [γ-^{32}P] ATP / ml), 1.25 mM $MnCl_2$, and a phosphoacceptor

substrate such as RCML. Reactions were initiated by the addition of ATP. Following incubation for periods up to 60 min, a 20μL portion of the reaction mixture was spotted onto a 2 x 2 cm square of Whatman 3MM paper and immediately immersed in a solution of 10% (w/v) trichloroacetic acid (TCA) containing 4% (w/v) sodium pyrophosphate ($NaPP_i$). The mixture was stirred for 20 min, the free liquid decanted, and the process repeated three more times using 5% (w/v) TCA containing 2% (w/v) $NaPP_i$ for periods of 20 min to overnight for each cycle. The filter papers were dried and then immersed in scintillation vials containing 1 ml of ScintiSafe Plus 50% scintillation fluid or 1 ml of ScintiSafe Plus 30% scintillation fluid. The quantity of [^{32}P] phosphate associated with the paper then was determined by liquid scintillation counting.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed essentially as described by Kamps and Sefton (Kamps and Sefton 1989). Radiolabeled proteins were isolated via SDS-PAGE and transferred to an Immobilon-P membrane as described above. The ^{32}P-labeled proteins were located by electronic autoradiography and the section of membrane to which they were bound excised and cut into approximately 1 mm x 1mm squares. The membrane squares were incubated in 100 μl of 6 N HCl for 1 hour at 100ºC. Following acid hydrolysis, the supernatant fluid was transferred to another tube and the liquid evaporated in a heat block at approximately 100ºC. The dried hydrolysate was then dissolved in 5 - 10 μl of pH 1.9 (50 ml 88% (v/v) formic acid, 156 ml glacial acetic acid, made up to 2 L with distilled water) or pH 3.5 (100 ml glacial acetic acid, 10 ml pyridine, made up to 2 L with distilled water) buffer by agitating on a vortex mixer.

Two-dimensional thin layer electrophoresis was used to resolve the phosphoamino acids in the hydrolysate. Xylene cyanole, 0.5 µl of 1 mg/ml stock solution was applied to the cellulose thin layer plate as tracking dye. By dividing the 20 x 20 x 0.1 cm plate into four 10 cm x 10 cm sections, up to four samples could be analyzed at one time. The TLC plate was divided into four quadrants, with a 2 cm border. A portion, 1 µl of a solution of 5 mM each phosphoserine, phosphothreonine, and phosphotyrosine was added to each sample and the mixture was applied, 0.5 µl at a time to avoid spreading. The samples were placed in the top right corner of each quadrant. Just prior to electrophoresis, the TLC plate was wetted by placing damp Whatman 3MM filter paper equilibrated in either pH 1.9 buffer (1st dimension) or pH 3.5 buffer (2nd dimension) on the TLC plate. The buffer was applied in this manner to help keep the samples concentrated.

Two-dimensional thin layer electrophoresis was conducted on a Multiphor II system using a cooling plate connected to a thermostatic circulator set at 7ºC. For the first dimension, pH 1.9, a potential of 500 volts was applied for a period of approximately 1.5 hours. The current limit was set at 100 mAmps and the power limit set at 200 watts. Afterwards the TLC plate was removed; air dried for at least 4 hours, turned 90º clockwise, and placed back on the Multiphor II plate. For the second dimension in pH 3.5 buffer, a potential of 500 volts was applied for 30 min. The current limit was set at 100 mAmps and the power limit set at 200 watts. The plate was dried with a hair dryer and the phosphoamino acid standards were visualized by spraying a fine mist of 0.2% (w/v) ninhydrin in acetone and baking for 5 min at 80ºC. The locations of the ^{32}P-labeled

phosphoamino acids from the protein samples were visualized by electronic autoradiography—using a Packard Instantimager.

CHAPTER III

CLONING AND EXPRESSION OF SULFOLOBUS SOLFATARICUS ORF SSO0443, PURIFICATION AND CHARACTERIZATION OF THE GENE PRODUCT: SSOPK5.

Analysis of *S. solfataricus* ORF *sso0433* and its protein product, SsoPK5

The nucleotide derived-amino acid sequence of ORF *sso0433* shown in Figure 3-1 displays many of the hallmarks of an atypical eukaryotic protein kinase. In order to determine whether *S. solfataricus* ORF *sso0433* encodes a functional protein kinase, the gene was cloned into a pET expression vector and its protein product expressed in *E. coli* BL21 (DE3) cells. The protein product will be referred to as SsoPK5.

Cloning ORF *sso0433* into pET29b/pET21d and expression of SsoPK5 in *E. coli*

The molecular cloning techniques used for both pET vectors were essentially the same, and the primers used in the polymerase chain reaction (PCR) protocol did not vary. ORF *sso0433*, the gene encoding SsoPK5, was amplified by PCR using *S. solfataricus* genomic DNA as template and two oligonucleotide primers (Figure 3-1) designed for the pET vector system as described in Materials and Methods (Chapter II). Amplification yielded a segment of DNA of the predicted size, 670 bp. The PCR product was ligated into the pET29b or pET21d expression vectors, which add sequences coding for fusion domains to facilitate affinity purification as illustrated in Figure 3-2, via their *NcoI* and *SalI* sites.

Figure 3-1. DNA derived amino acid sequence of SsoPK5

Shown above is the DNA sequence of *S. solfataricus* ORF *sso0433* and the protein it encodes, SsoPK5. The forward primer (SsoPK5 Forward) is shown in orange and the reverse primer (SsoPK5 Reverse) is shown in blue. In the oligonucleotides the restriction site sequences for *NcoI* (Forward primer) and *SalI* (Reverse primer) are underlined. The upstream and downstream regions of the DNA, to which the primers also anneal are not shown above.

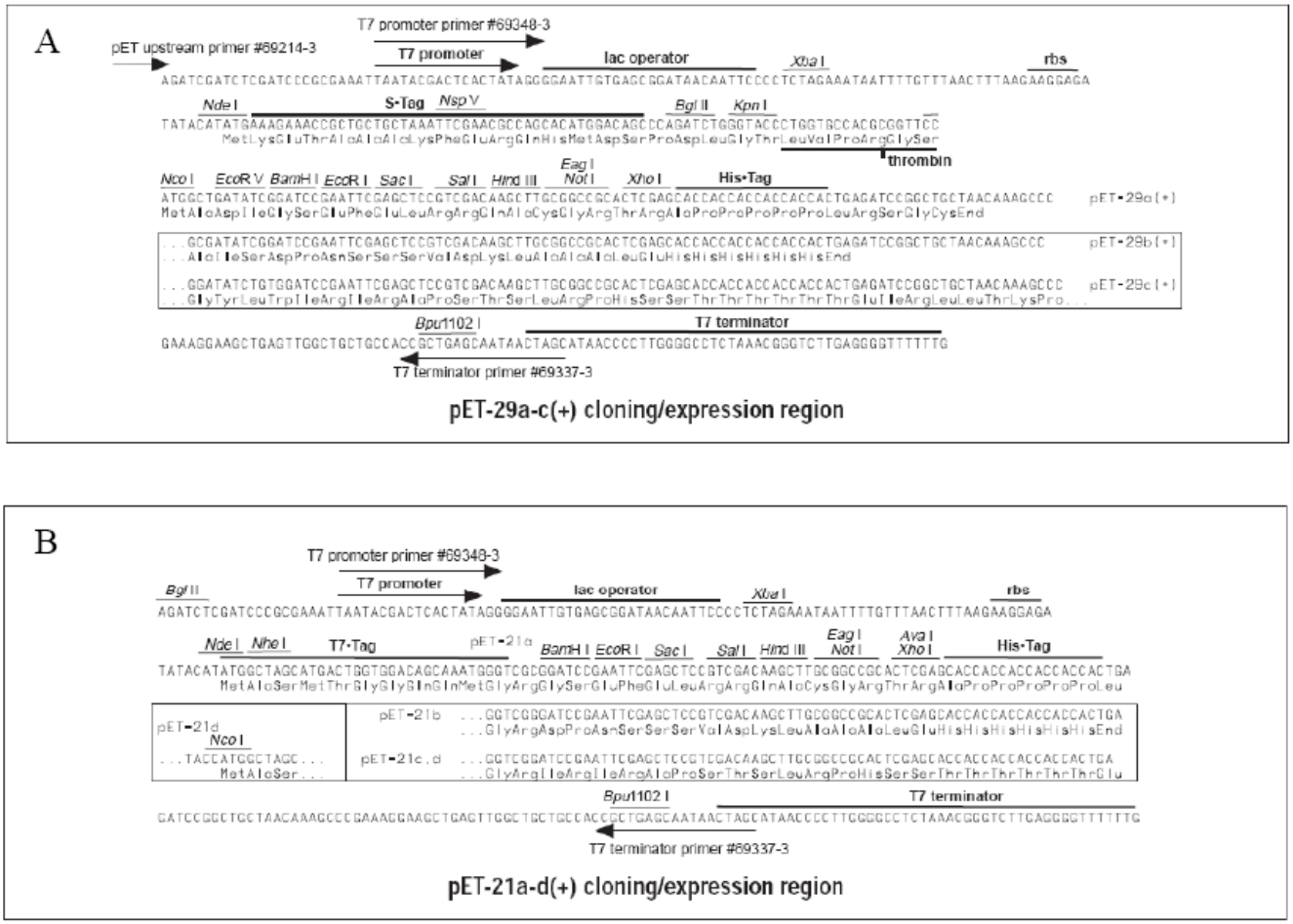

Figure 3-2. Diagrams of pET29b and pET21d vector

Important features of the vectors pET29b (A) and pET21d (B) are shown above. These include the sequences for the T7 promoter, T7 terminator, His-Tag, S-Tag, and the restriction sites within the multiple site cloning region of the vector. Because both vectors contained sites for the restriction enzymes, *NcoI* and *SalI*, identical primers were used for molecular cloning. This illustration is used with permission by EMD Chemicals Inc.

Recombinant plasmids were purified from the transformed α-select cells using QIAPrep Miniprep Kits. Sequencing in both the 5' – 3' direction (T7 promoter) and the 3' – 5' direction (T7 terminator) verified that the PCR amplified genomic DNA was indeed ORF *sso0433*, that no mutations had been introduced during PCR amplification, and that the inserts were ligated into the vector in the correct orientation. Next, the plasmids were transformed into the *E. coli* strain BL21 CodonPlus® (DE3) RIL, which contain extra copies of the three tRNA genes, *argU*, *ileY*, and *leuW*, that recognize rare codons for arginine (AGA and AGG), isoleucine (AUA), and leucine (CUA), respectively. The presence of these codons in heterologous genes can restrict the translation of proteins from organisms with A-T rich genomes, such as *S. solfataricus*, in ordinary *E. coli* strains.

Growth of transformed *E. coli* and induction of recombinant protein expression were carried out as described in Materials and Methods (Chapter II). It was observed that if the *E. coli* cells were incubated with the inducer, 1 mM IPTG, for more than 3 hours the yield of recombinant SsoPK5 tended to decrease.

Purification of SsoPK5 and preliminary assays for protein kinase activity

Both SsoPK5 recombinant fusion proteins were purified by immobilized metal affinity chromatography using the hexahistidine sequence as described in Materials and Methods (Chapter II). As *S. solfataricus* is an extreme thermophile, heat denaturation was tried as a means of enriching for SsoPK5 prior to affinity chromatography. Specifically, the soluble cell extract, was incubated for 10 min at 65°C, then subjected to centrifugation at 3,000 x g for 15 minutes at 4°C. The resulting supernatant liquid was

applied to a column of chelating Sepharose Fast Flow (1 ml column bed volume) that was charged with one volume of 0.1 M $NiSO_4$ as indicated by the manufacturer. The column was then washed as described in Materials and Methods (Chapter II). Fractions of 1 ml each were collected and analyzed for protein using SDS-PAGE. The fractions containing SsoPK5 alone (usually fractions 2 and 3) were pooled and retained. Following purification, the fractions were stored at either room temperature (~25°C), 4°C or at -80°C in 20% glycerol (v/v) to compare the efficacy of potential storage conditions. This technique was utilized for SsoPK5 with and without the N-terminal S-tag.

Recombinantly produced SsoPK5 was then assayed for phosphotransferase activity. Since it has been reported that the eukaryotic homologues of SsoPK5 phosphorylate acidophilic proteins *in vitro* (Facchin, Sarno et al. 2002), two proteins with an acidic isoelectric point—reduced, carboxyamidomethylated and maleylated (RCM) lysozyme and casein—were tested as potential phosphoacceptor substrates (Figure 3-3). The fractions stored at both 25°C and 4°C displayed the ability to transfer the γ-phosphate of ATP to RCM lysozyme and casein. However, no activity was detected for the fractions stored at -80°C. When stored for 10 days, we were unable to detect phosphotransfer activity in the samples kept at 4°C; however, we were still able to detect phosphotransferase activity in the fraction maintaining at room temperature.

At this point, we postulated that SsoPK5 was cold labile; therefore, we altered the purification procedure in an attempt to enhance specific activity and protein stability by performing all steps, with the exception of thermal denaturation and sonication, at room temperature. It was subsequently observed that protein purification by IMAC yielded no net increase in specific activity. Moreover, the resulting preparations displayed a marked

propensity to form precipitates. As the recombinant protein was relatively pure following thermal denaturation, IMAC was omitted.

CHARACTERIZATION OF SSOPK5

Assessment of protein kinase activity

The primary sequence alignment shown in Figure 3-4 illustrates the unexpectedly high sequence similarity between SsoPK5 and its eukaryotic counterparts. The amino acid residues highlighted in yellow represent specific subdomains characteristic of the ePKs. The underlined amino acid residues and the amino acid residues highlighted in red constitute the putative activation loop in Subdomain VIII. In many ePKs, phosphorylation of an amino acid residue in the activation loop stabilizes the protein kinase in the open confirmation, facilitating substrate binding and catalysis (Hanks and Hunter 1995). Overall, SsoPK5 and piD261/Bud32 share 49% amino acid sequence similarity and 28% identity. The corresponding percentages for SsoPK5 and PRPK are 57% and 32%. To identify potential functional differences and similarities between SsoPK5, piD261/Bud32, and PRPK, a thorough analysis of SsoPK5 phosphotransferase activity was conducted *in vitro*. Substrate preference, divalent metal ion preference, and optimal reaction conditions are just a few of the properties that affect the catalytic activity of SsoPK5.

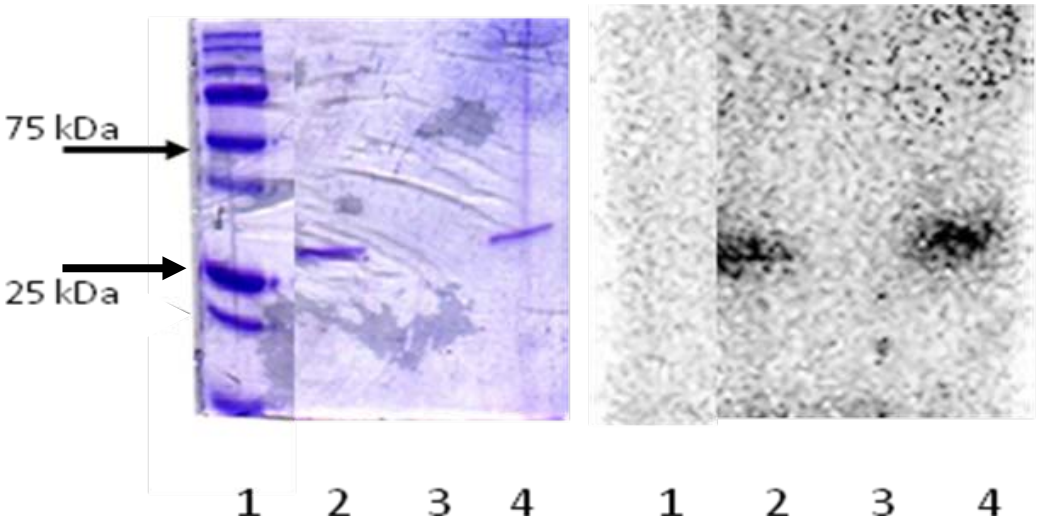

Figure 3-3. Preliminary phosphotransfer

SsoPK5 (5 μg) and casein (1 μg) were incubated with [γ-^{32}P] ATP with for 60 min at 65°C with as described in Materials and Methods (Chapter II). The incubation mixture was subjected to SDS-PAGE. The polyacrylamide gel was imaged with the Packard Instantimager then stained with Coomassie blue. Shown on the left is the Coomassie blue-stained gel with the positions and relative molecular masses of the protein standards indicated on the left. Shown on the right is an electronic autoradiogram of the same gel; here we observe ^{32}P radioactive transfer to both SsoPK5 and casein. The contents of each lane were as follows: Lane 1, Precision Plus Protein Standards (M_r listed at far left); Lanes 2, SsoPK5; Lane 3, casein; Lane 4, SsoPK5 plus casein.

Figure 3-4. Alignment of SsoPK5 with *Homo sapiens* p53 related protein kinase (PRPK) and *Saccromyces cerevisiae* piD261/Bud32.

Highlighted in yellow are the conserved amino acid residues in subdomain VIb; highlighted in red is the putative site (Threonine-151) for autophosphorylation in SsoPK5. The underlined residues correspond to the proposed activation loop.

Substrate preference

To determine the range of exogenous substrates SsoPK5 could phosphorylate *in vitro*, SsoPK5 was assayed using a filter paper assay. Casein, RCM lysozyme, myelin basic protein (MBP), and mixed histones were tested as potential phosphoacceptor substrates. As seen in Table 3-1, SsoPK5 was able to phosphorylate both RCM lysozyme and casein, but not MBP or mixed histones.

The filter paper assay measures the ^{32}P radioactivity that precipitates onto a section of Whatman 3 MM paper to which a portion of the reaction mixture was applied and then immersed in a solution containing trichloroacetic acid. To ensure that the γ-phosphate of [$\gamma^{32}P$]-ATP was physically transferred to either RCM lysozyme or casein, and not just associated with the protein precipitate by some other means, the remainder of the reaction mixture was subjected to SDS-PAGE. As can be seen in Figure 3-3, when the ^{32}P radioactivity was visualized using electronic autoradiography, it was observed to co-migrate with casein.

Metal ion preference of SsoPK5

To assess the divalent metal ion requirement of SsoPK5, solution assays were performed as described in Materials and Methods (Chapter II) with the exception that, in place of the standard divalent metal ion cofactor, Mg^{2+} (magnesium chloride), Mn^{2+} (manganese chloride), Ca^{2+} (calcium chloride), Ni^{2+} (nickel sulfate), and Zn^{2+} (zinc chloride) were added to a final concentration of 1.25 mM. The assays were performed in 50 mM Bis-tris, pH 7.0. Controls in which EDTA was added to a final concentration of 1.25 mM in place of a divalent metal ion were performed in parallel. As shown in Figure

Phosphoacceptor substrate (1.0 mg/ml)	Specific activity (pmol/min•mg)	Replicates
Casein	42.4 ± 23.0	n = 2
RCM lysozyme	14.9 ± 5.6	n = 6
Histones	n.d.	n = 1
MBP	n.d.	n = 3

Table 3-1. SsoPK5 phosphorylates acidophilic proteins *in vitro*.

The capacity of SsoPK5 to phosphorylate several exogenous proteins was examined using the filter paper methods described in Materials and Methods (Chapter II). Shown are the quantities of phosphate transferred to the listed proteins.

n.d. = no activity detected greater than either substrate or SsoPK5 alone

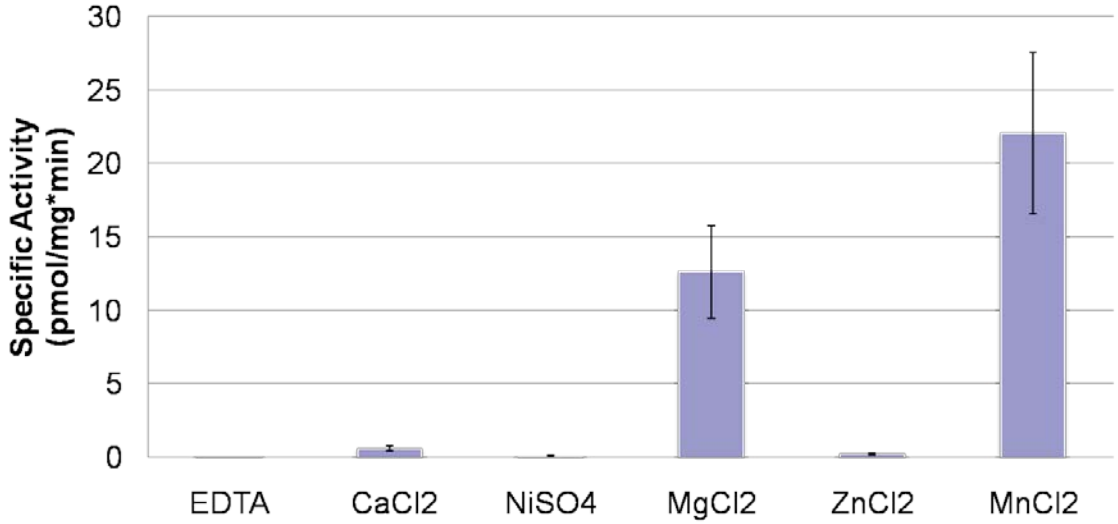

Figure 3-5. SsoPK5 phosphotransferase activity with several divalent metal ions

The phosphotransferase activity of SsoPK5 was assayed in the presence of the indicated divalent metal ions at 65C for 1 hour. Conditions were as described in Materials and Methods (Chapter II) with the exception that, in the place of the standard divalent metal, Mn^{2+}, the compound listed above were added at a final concentration of 1.25 mM. Shown is the average of duplicate determinations plus or minus the standard error.

3-5, analysis of the cofactor requirements at 65°C revealed a strong preference for Mn^{2+} over the other divalent metals surveyed. This preference mirrors that of *S. cerevisiae* piD261/Bud32 protein kinase, which also prefers Mn^{2+} as the cofactor *in vitro* (Stocchetto, Marin et al. 1997). However, PRPK, like many ePKs, utilizes Mg^{2+} (Abe, Matsumoto et al. 2001). It is important to note that many of the protein kinases isolated from *S. solfataricus* also prefer Mn^{2+} over Mg^{2+} (Lower and Kennelly 2003; Lower, Potters et al. 2004).

Ionic strength

In previous assays, the quantity of protein phosphorylated produced varied quite broadly between triplicates. In an effort to reduce variability, we investigated whether SsoPK5 was sensitive to the ionic strength of the assay solution. Several assays were performed to which NaCl and KCl were added. These results, depicted in Figure 3-6, indicated that added KCl or NaCl did not have a significant effect.

Optimization of pH for phosphotransferase activity

Optimal growth conditions for *S. solfataricus* are pH 2.0 and a temperature of 70°C. However, members of the genus *Sulfolobus* maintain an internal pH around 6.0 (Lubben and Schafer 1989). SsoPK5 exhibited phosphotransferase activity across a broad range of pH values; with a maximum around pH 7.0. Similar levels of activity were obtained in both 2-[Bis (2-hydroxyethyl) imino]-2-(hydroxymethyl)-1, 3-propanediol (Bis-tris) and tris(hydroxymethyl) aminomethane (TRIS) buffers. However,

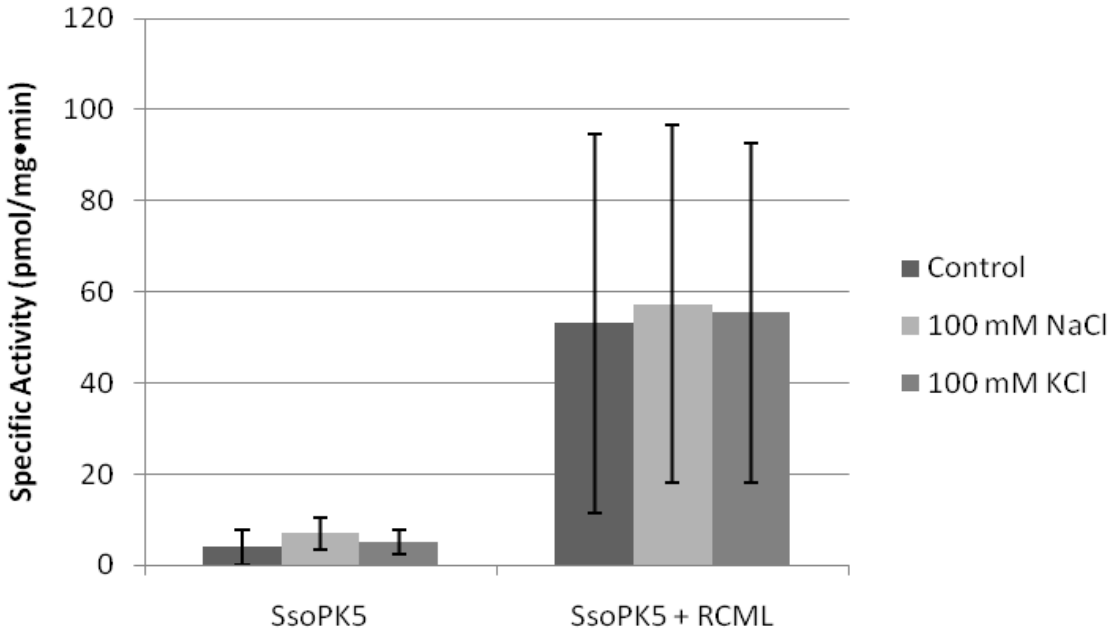

Figure 3-6. Ionic strength does not affect SsoPK5 phosphotransferase activity

The phosphotransferase activity of SsoPK5 was assayed in the presence of the indicated salt solution at 65°C for 60 min. Conditions were as described in Materials and Methods (Chapter II) with the exception that 100 mM NaCl or 100 mM KCl were added to each assay mixture. Shown is the average of duplicate determinations plus or minus the standard error.

Bis-tris was chosen over Tris due to the limited buffering capacity of TRIS at pH 7.0. Disappointingly, the change in buffer and pH yielded no improvement in the consistency between replicate assays.

SsoPK5 phosphorylates p53

Both PRPK and piD261/Bud32 phosphorylate *Homo sapiens* p53 *in vitro*. (Yeast lack a recognizable homolog of p53 (Facchin, Lopreiato et al. 2003).) An obvious question was whether SsoPK5 would be capable of transferring the γ-phosphate from ATP to p53, specifically to a p53-GST fusion protein purchased from Santa Cruz Biotechnologies. At first glance it did not appear that SsoPK5 was capable of phosphorylating the p53-GST fusion protein; however, it had been reported that the phosphotransferase activity of PRPK required pre-incubation with a COS-7 cell lysate (Abe, Matsumoto et al. 2001). Therefore, we postulated that SsoPK5 might also require activation. Since PRPK has been speculated to play a role in gene expression, we tested salmon sperm DNA as a potential activator for SsoPK5. As can be seen in Figure 3-7, SsoPK5 did in fact phosphorylate the fusion protein, p53-GST, when Salmon sperm DNA was present in the assay.

Next, we asked whether SsoPK5 was in fact transferring the γ-phosphate of ATP to the p53 portion of the fusion protein. Samples of SsoPK5 with and without p53-GST were incubated at 65°C for 30 minutes. The 50 μL reaction mixture was divided; 20 μL was utilized for a solution assay to measure protein kinase activity; 15 μL was loaded onto a 12% (w/v) SDS-PAGE gel that was subsequently stained with Coomassie; 15 μL was loaded onto a 12% (w/v) SDS-PAGE gel, which upon completion of SDS-PAGE, the

sample was then transferred to an Immobilon-P membrane, as described in Materials and Methods (Chapter II).

To determine whether SsoPK5 had transferred the γ-phosphate of ATP to the p53 portion of the fusion protein, the membrane bound protein was tested for immunoreactivity toward anti-phospho-p53 antibodies from Cell Signaling Technology directed against six previously identified phosphorylation sites on p53: Ser-6, Ser-15, Ser-20, Ser-37, Ser-46, and Ser-392. As shown in Figure 3-9, p53-GST that has been phosphorylated by SsoPK5 *in vitro* bound two of the antibodies, those directed against P-Ser-15 and P-Ser-20. It would thus appear that SsoPK5 phosphorylated p53-GST at these sites within the p53 domain.

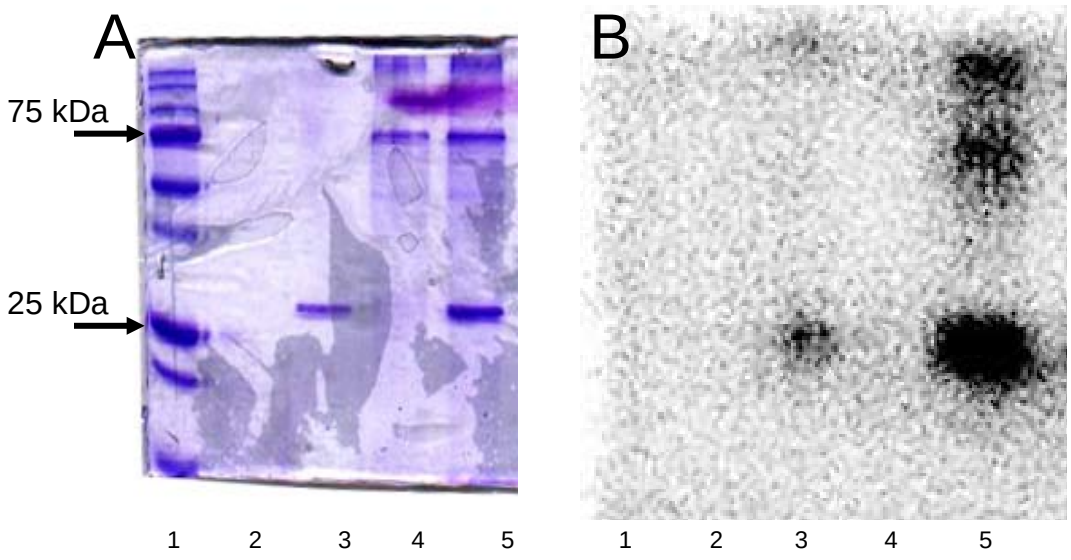

Figure 3-7. SsoPK5 phosphorylates itself and p53-GST when incubated with [γ-^{32}P] ATP *in vitro*.

SsoPK5 (5 μg), p53 (5 μg) and 0.2 mg/ml salmon sperm DNA were incubated [γ-^{32}P] ATP with for 60 min at 65°C with as described in Materials and Methods (Chapter II). The incubation mixture was subjected to SDS-PAGE. The polyacrylamide gel was imaged with the Packard Instantimager then stained with Coomassie blue. Shown on the left (A) is the Coomassie blue-stained gel with the positions and relative molecular masses of the protein standards indicated on the left. Shown on the right (B) is an electronic audioradiogram of the same gel; here we observe ^{32}P transfer to both SsoPK5 and p53. The contents of each lane were as follows: Lane 1, Precision Plus Protein Standards (M_r listed at far left); Lane 2, empty; Lane 3, SsoPK5; Lane 4, p53-GST; Lane 5: SsoPK5 plus p53-GST.

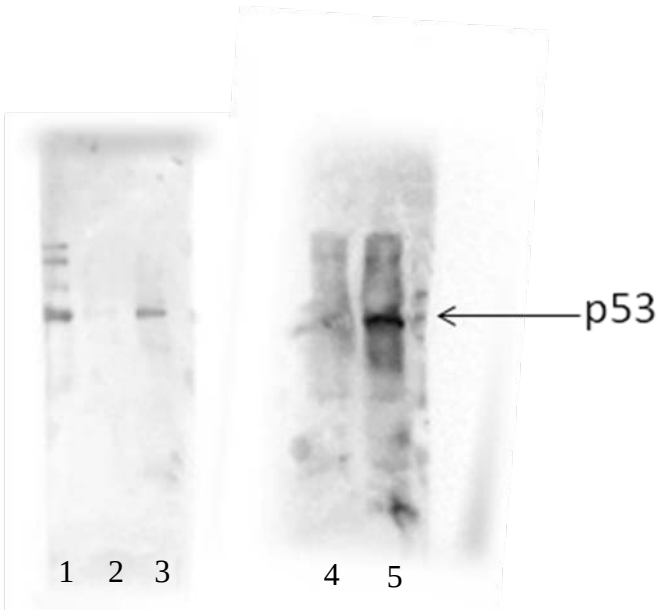

Figure 3-8. SsoPK5 phosphorylates p53 on Ser-15 and Ser-20.

SsoPK5 (5 μg), p53 (5 μg) and 0.2 mg/ml salmon sperm DNA were incubated [γ-^{32}P] ATP with for 60 min at 65°C with as described in Materials and Methods (Chapter II). The incubation mixture was subjected to SDS-PAGE. The proteins were transferred from the polyacrylamide gel to a Immobolin-P PVDF membrane as described in Materials and Methods (Chapter II). Shown on the left is as image of the PVDF developed with the anti-phosphos-Ser-20 antibody. Shown on the right is an electronic image of the PVDF membrane developed with the antipphospho-Ser-15 antibody; here we observe that p53 is phosphorylated on Ser-15 and Ser-20. The contents of each lane were as follows: Lane 1, Precision Plus Protein Standards; Lane 2, SsoPK5; Lane 3, SsoPK5 and p53-GST; Lane 4, SsoPK5; Lane 5, SsoPK5 and p53-GST

CHAPTER IV

SSOPK5 VARIANT ACTIVITY AND ACTIVATION

Analysis of SsoPK5 variants and autophosphorylation

The yeast homologue of SsoPK5, piD261/Bud32, phosphorylates itself on Ser-184, Ser-187, or both (Facchin, Lopreiato et al. 2002). Recent research on another piD261/Bud32 subfamily protein kinase in *M. jannaschii* indicates that it too autophosphorylates, although the specific amino acid residue(s) modified remain(s) unknown (Hecker, Lopreiato et al. 2008). Does SsoPK5 possess the ability to phosphorylate itself? If so, can we identify the specific phosphoacceptor amino acid residues?

Autophosphorylation of SsoPK5

Like many other protein kinases, SsoPK5 possesses the capacity to autophosphorylate. Interestingly, preliminary data suggested that autophosphorylation was linked to the phosphorylation of exogenous protein substrates. Only trace levels of ^{32}P radioactivity were incorporated into SsoPK5 when the protein kinase was incubated with [γ-^{32}P] ATP alone. However, the level markedly increased in mixtures where the phosphoacceptor substrates casein, RCM lysozyme, or p53 also were present (Figure 3-7). However, no enhancement of autophosphorylation was observed when proteins, such as histones and BSA, that were not phosphoacceptors, were added. In order to determine the types of residues phosphorylated, phosphoamino acid analysis was conducted. This

analysis revealed that autophosphorylation took place on both serine and threonine residues (Figure 4-1). We next asked which particular serine or threonine residues might be phosphorylated.

SsoPK5 threonine-151 is an important residue for phosphotransferase activity

Knowing that serine and threonine residues were phosphorylated on SsoPK5, we examined its amino acid sequence for clues to potential autophosphorylation sites using its eukaryotic homologues as templates. The reported autophosphorylation sites of piD261/Bud32, Ser-184 and/or Ser-187, were used as guides (Facchin, Lopreiato et al. 2002). The closest match to these residues in SsoPK5 was Thr-151, which aligns with Ser-184 of piD261/Bud32. A serine is conserved at this position in PRPK, although it has yet to be determined whether this residue is modified through autophosphorylation (Figure 3-3).

Site-directed mutagenesis was used to determine if Thr-151 in SsoPK5 was autophosphorylated. The following variants of SsoPK5 were produced via site-directed mutagenesis and recombinant protein expression as described in Materials and Methods (Chapter II): Thr-151 to Ala-151 (T151A) and SsoPK5 Thr151 to Asp-151 (T151D). These variants were purified and stored as described in Chapter III, then assayed for phosphotransferase activity. As would be expected if Thr-151 were targeted by autophosphorylation, substitution of Thr-151 in SsoPK5 by either alanine or aspartate reduced the level of [^{32}P] incorporated into SsoPK5 following autophosphorylation to less than half that of the wild type enzyme. While the alanine-substituted version

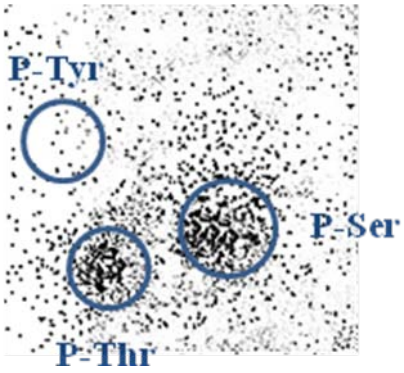

Figure 4-1. SsoPK5 phosphorylated serine and threonine residues *in vitro*

SsoPK5, 8.75 μg, was incubated with [γ-^{32}P] ATP and RCM lysozyme as described in Materials and Methods (Chapter II). The polypeptides within each reaction mixture were separated by SDS-PAGE and transferred to an Immobolin-P PVDF membrane. The section of the membrane containing SsoPK5 was excised and incubated in 6 N HCl for 1 hour at 100°C. The hydrolysates were spotted on to a thin layer chromatography plate then the plate was subjected to two-dimensional thin layer electrophoresis. Shown is the audioradiogram of the thin-layer chromatographic plate, with the positions of the phosphoamino acid standards circled: P-ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

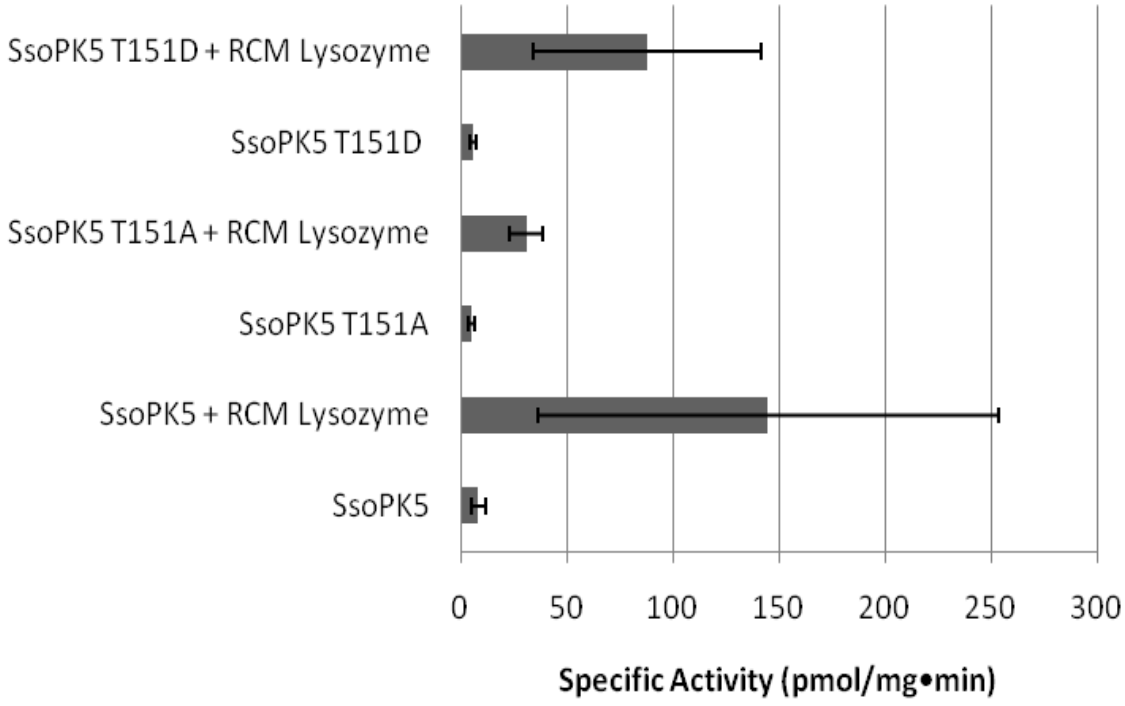

Figure 4-2. SsoPK5, SsoPK5 T151A, and SsoPK5 T151D specific activities vary given the same conditions in an *in vitro* activity assay.

The activity of SsoPK5 and the mutationally-altered variants were determined by the filter paper assay using RCM lysozyme as substrate. Assays were performed for 60 min at 65ºC. Shown is the average specific activity, plus or minus mean average deviation, for triplicate determinations in the presence and absence of RCM lysozyme.

exhibited much-reduced specific activity relative to wild type SsoPK5, the activity of the aspartate-substituted version, which introduces a potential negatively charged group at this position, was comparable to that of the unmodified enzyme (Figure 4-2).

Is SsoPK5 activated by nucleotides or related species?

Protein kinase activity is highly regulated in the cell. One common mechanism by which the phosphotransferase activity of some protein kinases can be affected is via the binding of small molecules, such as cAMP in the case of cAMP-dependent protein kinase A (Skalhegg and Tasken 1997). It has been reported that it is essential to pre-incubate recombinant PRPK with a COS7 cell extract in order for phosphotransfer to be observed *in vitro* (Abe, Matsumoto et al. 2001). Given that piD261/Bud32 subfamily protein kinases may be involved in transcriptional regulation and telomerase regulation (Downey, Houlsworth et al. 2006; Kisseleva-Romanova, Lopreiato et al. 2006), we hypothesized that SsoPK5 might be regulated by a nucleotide or related species. Preliminary experiments had indicated that salmon sperm DNA activated SsoPK5 *in vitro*. Additionally, we postulated that activators might stabilize SsoPK5, and would thus be expected to reduce the extreme variability between the replicates *in vitro*.

The addition of salmon sperm DNA stimulated the protein kinase activity of SsoPK5 several-fold. Activation with salmon sperm DNA was maximal around 0.01 µg/µl, and then fell off at higher concentrations — possibly through competition for the binding of the acidic protein substrate or inhibition of ATP binding by any free nucleotides present in the preparation (Figure 4-3). Two other polyanionic compounds

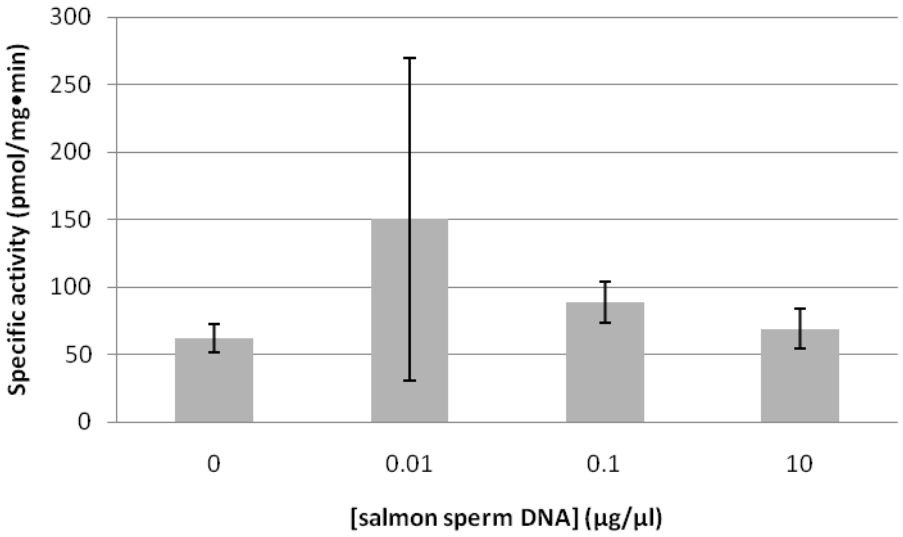

Figure 4-3. SsoPK5 is activated by salmon sperm

The activity of SsoPK5, toward RCM lysozyme was assayed as described in Materials and Methods (Chapter II) except in this case varying concentrations of salmon sperm DNA were added to the assay mixtures. SsoPK5 is activated by salmon sperm DNA at 0.01 µg/µl and 0.1 µg/µl. Shown are the averages for triplicate assays plus or minus standard error.

poly(ADP-ribose) also stimulated the activity of SsoPK5 *in vitro.* However, heparin and RNA did not. Moreover, when alternate DNA preparations were tested, it was observed that commercial preparations of genomic DNA from *S. solfataricus* or *Thermoplasma volcanium* were generally stimulatory, a commercially available DNA plasmid did not. Treatment with restriction endonucleases to transform it from a circular to linear configuration had no effect.

As both the plasmid DNA and RNA preparations had been isolated in the laboratory and were therefore of known purity, it appeared likely that the stimulatory potency of commercial DNA preparations and commercial poly(ADP-ribose) was derived from some component other than the polymers themselves—possibly some hydrolytic breakdown product. This hypothesis was supported by the observation that 5'-AMP, originally included as a mononucleotide control in our preliminary experiments, also stimulated the protein kinase activity of SsoPK5 (Figure 4-5). We therefore surveyed a broad spectrum of mononucleotides, dinucleotides, and related compounds for their capacity to stimulate the catalytic activity of SsoPK5. Table 4-1 lists compounds tested. From this extensive search only one additional compound, ADP-ribose, was observed to noticeably stimulate the phosphotransferase activity of SsoPK5 (Figures 4-4 and 4-5). Relative to 5'-AMP, ADP-ribose was demonstrably more potent, stimulating the catalytic activity of SsoPK5 by several-fold at concentrations as low as 10 nM, a concentration several orders of magnitude below the range at which 5'-AMP was effective. Activation also proved highly specific. For example, neither 5'-ADP nor cyclic ADP-ribose was stimulatory at the concentrations tested, i.e. 2 μM and 100 μM.

Activators	Non-activators
Genomic DNA, *S. solfataricus*	Plasmid pET29b, circular
Genomic DNA, *Thermoplasma volcanium*	Plasmid pET29b, treated with *NcoI* and *SalI*
Salmon sperm DNA	RNA, *Anopheles stephensi*
Poly(ADP-ribose)	Heparin
5'-AMP	ADP
ADP-ribose	Diadenosine tetraphosphate
	Cyclic ADP-ribose
	Adenosine
	Phosphoribsoyl pyrophosphate
	Sucrose tetraphosphate
	Ribose 5-phosphate
	Fructose 6-phosphate
	FAD
	FADH
	NAD
	NADH
	NADP
	NADPH
	Coenzyme A, reduced
	Coenzyme A, oxidized

Table 4-1. Potential nucleotide SsoPK5 activators surveyed

The activity of SsoPK5 toward RCM lysozyme was assayed in the presence of each of the compounds listed above. Nucleotides, dinucleotides, and their derivatives were tested at final concentrations of 2 µM and 100 µM with the exception of 5' – AMP and ADP-ribose, which were tested at 1 µM, 10 µM, 100 µM, 1 mM, and 10 mM. DNA was added to a final concentration of 1 µg/ml with the exception of *S. solfataricus* genomic DNA, which was test at 0.1 µg/ml and 7 µg/ml, and salmon sperm DNA, which was tested at 0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml and 10 µg/ml. Poly (ADP-ribose) was tested at final concentrations of 0.1 ng/ml, 1.0 ng/ml, 10 ng/ml and 100 ng/ml. Those compound observed to stimulate the protein kinase activity of SsoPK5 by greater than or equal to two-fold relative to a control with no additions were listed under "Activators". All other compounds were classified as "Non-activators".

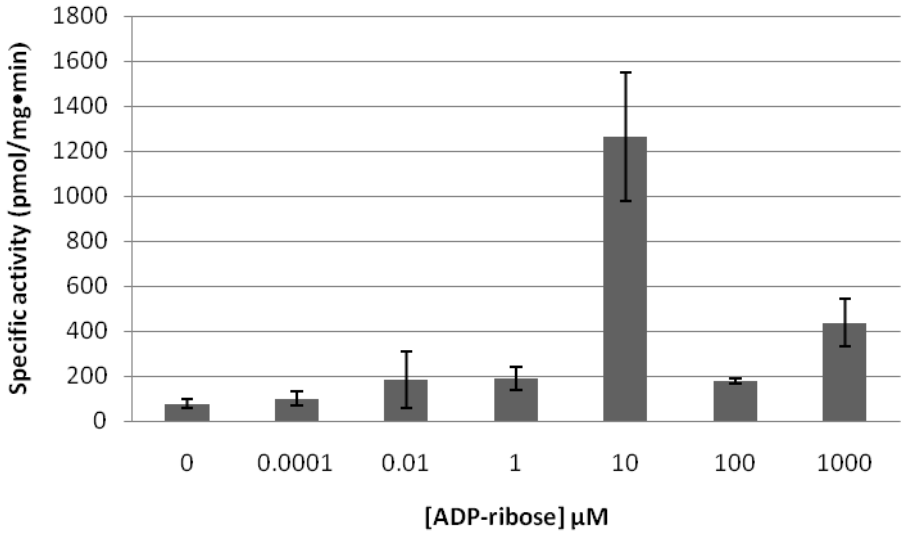

Figure 4-4. SsoPK5 is activated by ADP-ribose

The activity of SsoPK5, toward RCM lysozyme was assayed as described in Materials and Methods (Chapter II) with the exception that ADP-ribose added to the assay mixture to the indicated final concentrations. Shown are the averages for triplicate assays plus or minus standard error.

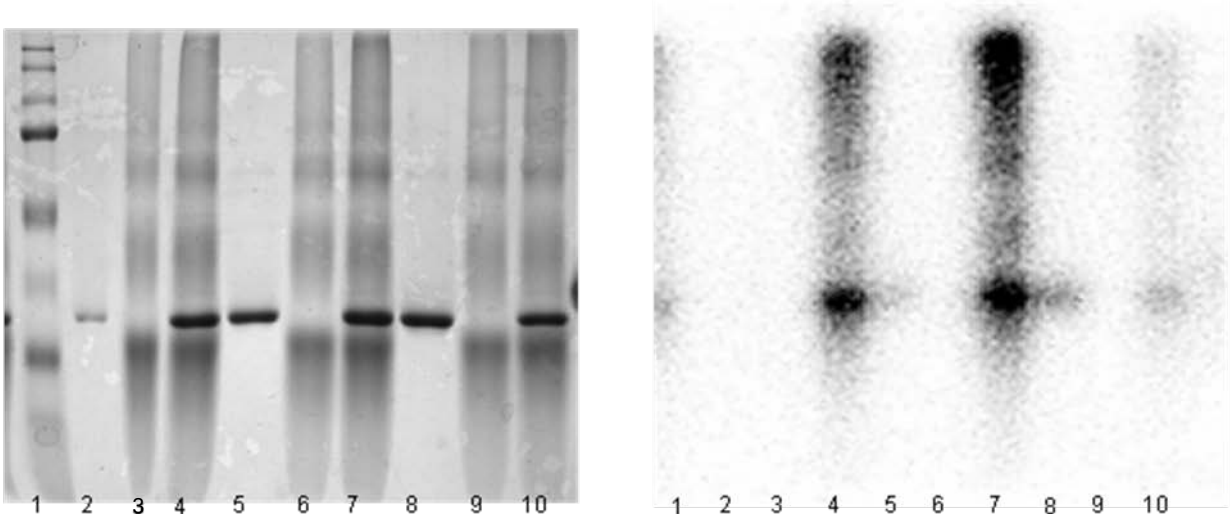

Figure 4-5. SDS-PAGE gel containing sample of SsoPK5, RCML, and varying concentrations of ADP-ribose and electronic audioradiogram of SDS-PAGE gel

SsoPK5 was incubated with RCM lysozyme (lanes 8-10) and either 100 µM AMP (lanes 2-4) or 2µM ADP-ribose (lanes 5-7) and [γ-^{32}P] ATP for 60 min at 65°C with as described in Materials and Methods (Chapter II). The incubation mixture was subjected to SDS-PAGE. Shown on the left is the Coomassie blue-stained gel with the positions and relative molecular masses of the protein standards indicated on the left. Shown on the right is an electronic audioradiogram of the same gel. The contents of each lane were as follows: Lane 1, Precision Plus Protein Standards (M_r listed at far left); Lane 2, SsoPK5; Lane 3, RCM lysozyme; Lane 4, SsoPK5 and RCM lysozyme; Lane 5: SsoPK5; Lane 6, RCM lysozyme; Lane 7, SsoPK5 and RCM lysozyme; Lane 8, SsoPK5; Lane 9, RCM lysozyme; Lane 10, SsoPK5 and RCM lysozyme.

Disappointingly, the presence of these activators had little impact on the extraordinary variability between replicates.

Stabilization of SsoPK5 activity by nucleotide effectors

While the average specific activity of SsoPK5 was reasonably consistent from experiment to experiment, dramatic differences (i.e. 50% or more from the mean) were routinely observed among the replicates in individual experiments. Attempts to reduce variability by testing a variety of buffers (Figure 4-6), manipulating pH, ionic strength, divalent metal cofactor, or purification techniques, varying the order of addition of the various components of the assay mixture, or via the addition of potential stabilizing agents such as bovine serum albumin, nonionic detergents, or glycerol proved unsuccessful (See Chapter III). Substituting glass tubes for the plastic ones normally used to conduct assays was also without effect (Figure 4-6). Therefore, we decided to explore other, more specific means for enhancing the stability of the enzyme.

A method of determining whether an enzyme is stable under a particular set of conditions is to pre-incubate it for varying times under those conditions and then testing for the level of activity remaining (Eisenthal and Danson 2002). When SsoPK5 and RCM lysozyme were pre-incubated in the assay mixture without ATP for 30 minutes at 65ºC the level of activity detected after pre-incubation was less than that in samples that had not been subject to pre-incubation (Table 4-2). We asked whether adding an activator, either ADP-ribose or 5'-AMP, to the pre-incubation mixture would stabilize the enzyme. As shown in Table 4-2, the presence of an activator during pre-incubation of

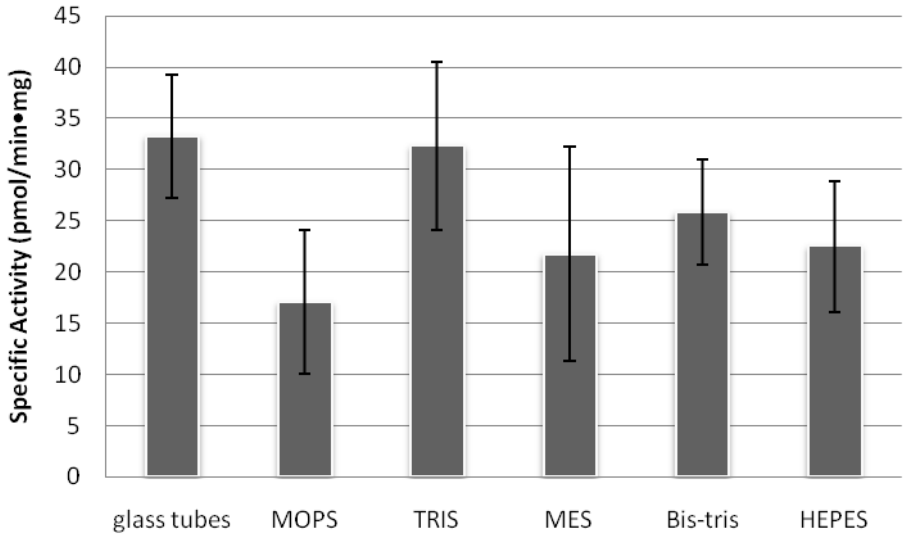

Figure 4-6. Effect of buffer and tube material on the phosphotransferase activity of SsoPK5

SsoPK5 was assayed with RCM lysozyme as phosphoacceptor substrate for 60 min at 65°C under standard conditions with the exception that either MOPS pH 7.0, TRIS pH 7.0, MES pH 6.5, or HEPES pH 7.0 was substituted for Bis-tris pH 7.0, or virgin glass tubes were substituted for plastic microcentrifuge tubes. Shown is the average of triplicate assays for each condition plus or minus standard error.

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Pre-incubation				Fold Activation
SsoPK5	RCM lysozyme	AMP	ADP	
-	-	-	-	1.00
+	+	-	-	n.d.
+	+	+	-	1.42 ± 0.41
+	-	-	-	1.03 ± 0.28
+	-	+	-	2.30 ± 0.19
+	-	-	+	0.91 ± 0.21

Table 4-2. Pre-incubation with RCM lysozyme and potential effectors

SsoPK5 was pre-incubated with RCM lysozyme and indicated nucleotides if noted, then assayed for protein kinase activity toward RCM lysozyme as described in Materials and Methods (Chapter II). The concentrations of each of the nucleotides present during the pre-incubation were 100 µM with the exception of ADPR, which was present at 2 µM. Shown are the average of duplicate determinations, normalized to the activity measured without pre-incubation (150.5 ± 16.4 pmol/mg •min), plus or minus standard error. The abbreviations used include reduced carboxyamidomethylated and maelylated, RCM; ADP-ribose, ADPR; 5'-AMP, AMP; n.d., no detectable activity.

SsoPK5 with RCM lysozyme for 30 min at 65°C both increased the amount of activity recovered following pre-incubation and reduced the level of variability. RCM lysozyme, the phosphoacceptor protein substrate, was initially included in the pre-incubation mixture; however, subsequent phosphotransferase assays revealed that similar results were obtained if it was omitted at this stage (Table 4-2).

Were 5'-AMP or ADP-ribose simply acting as mimics of the phosphodonor substrate, ATP? If so, 5'-ADP would be expected to exhibit similar effects. As shown in Table 4-2, ADP had no effect. We next asked whether ATP might further stabilize the enzyme. In order to do so, we omitted the phosphoacceptor protein from the pre-incubation mixture. As shown in Table 4-3, pre-incubating SsoPK5 with both 2 μM ADP-ribose and 10 μM ATP increased the amount of activity recovered and decreased the variability between replicates.

Since the addition of ATP to the pre-incubation mixture had a noticeable effect, we considered the possibility that the activators stimulated the autophosphorylation of SsoPK5 during pre-incubation. In order to observe if phosphotransfer was taking place, [γ^{-32}P] ATP was used for the pre-incubation and the samples subject to SDS-PAGE. The resulting electronic autoradiogram revealed no incorporation of ^{32}P radioactivity SsoPK5 during the pre-incubation. While SsoPK5 did not autophosphorylate during pre-incubation, this did not rule out a mechanism dependent upon the hydrolysis of ATP. Therefore, two analogues of ATP that are resistant to hydrolysis, β, γ-imido-triphosphate (AMP-PNP) and β, γ-methyleneadenosine 5′-triphosphate (AMP-PCP), were tested for their ability to mimic the effects of ATP (Figure 4-7). Pre-incubation of SsoPK5 either

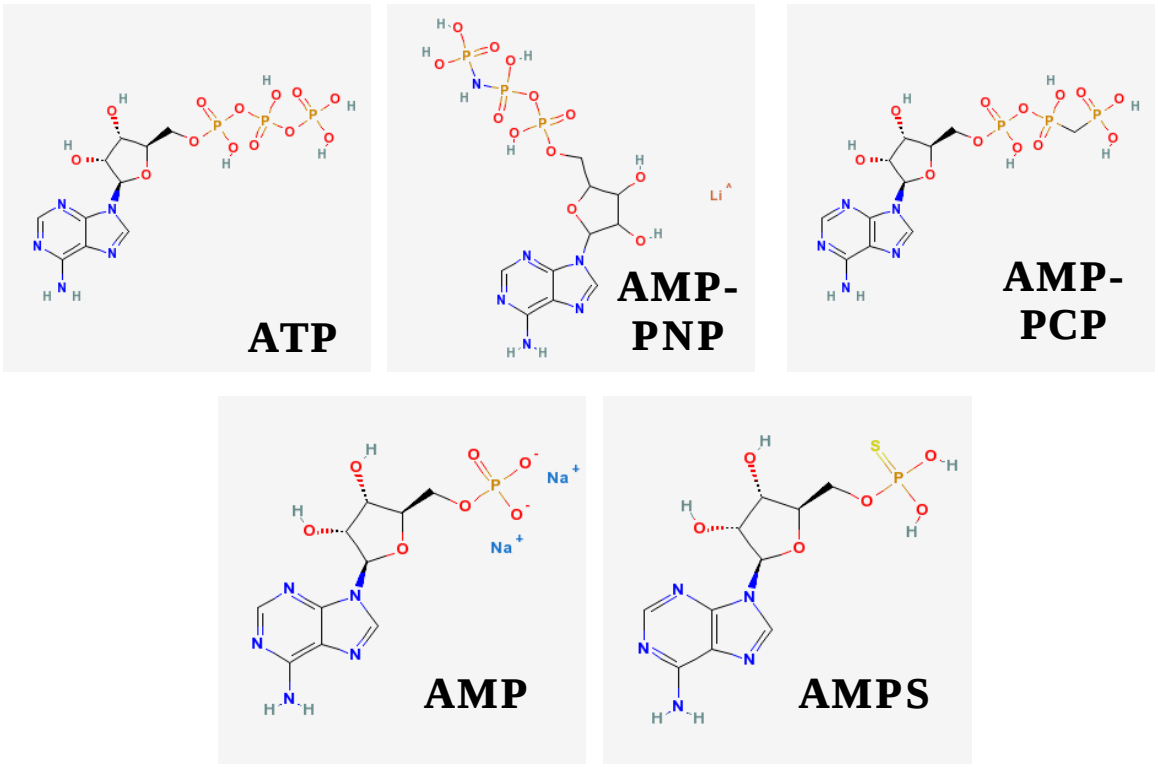

Figure 4-7. Structures of AMP, ATP, and the ATP analogs

AMP-PCP (β,γ-methylene adenosine 5'-triphosphate), AMP-PNP (adenylyl-imidodiphosphate), AMP (5'-AMP), and AMPS (adenosine 5'-O-thiomonophosphate) molecular structures

Structural representation of the nucleotides are available from National Center for Biotechnology Information.

Pre-incubation					Relative Activity	n
ATP	AMPPCP	ADPR	AMP	AMPS		
-	-	-	-	-	1.00	4
+	-	-	-	-	1.42 ± 0.31	4
+	-	+	-	-	2.79 ± 0.81	4
-	+	-	-	-	1.66 ± 0.37	2
-	+	+	-	-	9.63 ± 4.37	2
+	-	-	+	-	2.61 ± 0.28	2
+	-	-	-	+	2.95 ± 0.15	2
-	+	-	+	-	3.14 ± 0.17	2
-	+	-	-	+	3.10 ± 0.13	2

Table 4-3. SsoPK5 phosphotransferase activity toward RCM lysozyme in the presence of various nucleotides

SsoPK5 was pre-incubated with the indicated nucleotides then assayed for protein kinase activity with RCM lysozyme as a substrate as described in Materials and Methods (Chapter II). The concentrations of each of the nucleotides present during the pre-incubation were 10 µM with the exception of AMP and AMPS, which were present at 100 µM. Shown are the average of replicate determinations, normalized to the activity measured in the absence of nucleotides during pre-incubation (348.5 pmol /min•mg ± 143.0), plus or minus standard error. The abbreviations used include β,γ-methylene adenosine 5'-triphosphate, AMPPCP; ADP-ribose, ADPR; 5'-AMP, AMP; and adenosine 5'-O-thiomonophosphate; AMPS.

with 10 μM AMP-PNP or 10 μM AMP-PCP reduced our standard error among the replicates, including those with activator, as well if not better than ATP itself (Table 4-3).

Does AMP serve as a phosphoryl donor during pre-incubation?

As described previously, activation was time-dependent, generally requiring a pre-incubation period of thirty minutes to produce maximum effect. Having determined that hydrolysis of the β, γ-pyrophosphate bond in ATP was not necessary for the stabilizing effect of ATP, we wished to determine whether hydrolysis of the phosphoester bonds in 5'-AMP or ADP-ribose underlay their effects on SsoPK5. In order to test this, we took advantage of the fact that the phosphoester bond in adenosine 5'-O-thiomonophosphate (AMPS), an analog of 5'-AMP in which one of the phosphoryl oxygens is replaced by sulfur, is nearly two orders of magnitude more resistant to hydrolysis than that of 5'-AMP (Murray and Atkinson 1968). If phosphoester bond hydrolysis is necessary for activation, it would be expected that AMPS would be a poor or completely ineffective substitute for 5'-AMP. When AMPS was tested for its activation potential, it was observed to stimulate the activity of SsoPK5 in a manner comparable to that of 5'-AMP (Table 4-3). Moreover, the combination of AMPS and AMP-PCP proved as effective as 5'-AMP plus ATP.

CHAPTER V

DISCUSSION

Researchers have discovered archaeal molecular fossils ("biomarkers") dating as far back as 2.5 billion years (Brocks, Logan et al. 1999). Thus, the members of the domain *Archaea* inhabited the earth a mere one billion years after its formation, implicating them as the forerunners of eukaryotic life on this planet, including humans. The overall goal of the research described herein was to map a portion of the phosphorylation network in the archaeon *S. solfataricus* by examining SsoPK5, a primordial protein kinase found in all eukaryotes and archaeons.

In the *Eukarya,* the vast majority of protein kinases are characterized by the presence of a basic amino acid within subdomain VIb. The nature of this amino acid has been a reliable indicator of whether a protein kinase was specific for serine/threonine resides or tyrosine residues. With the exception of the protein products of ORF *sso2291*, ORF *sso3207,* and *sso3182,* the putative protein kinases encoded by the genome of *S. solfataricus* do not contain either a lysine or an arginine in subdomain VIb (Kennelly 2003). One example is SsoPK5, which is encoded by ORF *sso0433*. Alignment of the amino acid sequence of SsoPK5 with its eukaryotic counterparts (Figure 3-3) revealed unexpectedly high sequence similarity with the piD261/Bud32 subfamily of protein kinases, thus leading us to posit that these protein kinases share functional in addition to sequence homology.

Can we stabilize SsoPK5 by altering the vector and/or purification techniques?

Why was ORF *sso0433* cloned into two expression vectors, pET29b and pET21d? The construct made with pET29b yielded an active product. However, the sequences of its S-Tag and thrombin cleavage site include two threonine and two serine residues. In an effort to avoid the complications inherent in the possible adventitious phosphorylation of these residues it was decided that we would clone ORF *sso0433* into a different vector, pET21d, which does not code for an N-terminal S-Tag. Unfortunately, SsoPK5 expressed using this vector lacked detectable phosphotransferase activity. We therefore reverted to expressing SsoPK5 using the pET29b vector.

We determined that SsoPK5 was cold labile. The catalytic activity of SsoPK5 did not tolerate storage at -80°C. When a sample was stored at 4°C for 10 days, a precipitate formed, this was accompanied by a loss of phosphotransferase activity. The sample kept at room temperature retained activity. Since *S. solfataricus* typically resides at temperatures ranging from 60°C – 90°C (Lubben and Schafer 1989; Giaquinto, Curmi et al. 2007), the idea that SsoPK5 was not tolerant of temperatures below 25°C was reasonable. Therefore, it was decided to isolate and store SsoPK5 at room temperature.

Unfortunately, precipitates were soon observed in this material as well. The metals leaching from the IMAC column were suspected as contributors to this. Since neither the purity nor the specific activity of SsoPK5 was noticeably enhanced by IMAC, this step was omitted. Not only did this eliminate the formation of precipitates upon storage, the final yield increased. Unfortunately, this did not also eliminate the observed variability in SsoPK5 phosphotransferase activity between replicates.

SsoPK5 displays both structural and functional parallels with other members of the piD261/Bud32 family of protein kinases.

SsoPK5 exhibits significant functional similarities to its eukaryotic homologues. Like piD261/Bud32 from *S. cerevisiae* (Facchin, Sarno et al. 2002), SsoPK5 phosphorylated acidophilic proteins such as casein and RCM lysozyme *in vitro*. Unlike nearly all ePKs characterized to date, including PRPK, SsoPK5 preferred Mn^{2+} over Mg^{2+} as a cofactor (Abe, Matsumoto et al. 2001; Kennelly 2003). Intriguingly, the exceptions to this pattern include piD261/Bud32 (Stocchetto, Marin et al. 1997; Facchin, Lopreiato et al. 2002) as well as two other ePKs of archaeal origin—SsoPK2 and SsoPK3 (Lower and Kennelly 2003; Lower, Potters et al. 2004). Moreover, SsoPK5 phosphorylated p53-GST *in vitro*, like both PRPK and piD261/Bud32 protein kinases. Western blots indicated that SsoPK5 phosphorylated p53 on both Ser-15 and Ser-20, but not Ser-37 or Ser-392. It is noteworthy that Ser-15 is also phosphorylated by piD261/Bud32 and PRPK. Phosphorylation of p53 at Ser-15 is upregulated in response to DNA damage and leads to p53 stabilization and increased activity. It is suspected that PRPK may regulate apoptosis and cell cycle arrest by phosphorylating p53 on Ser-15 *in vivo* (Abe, Matsumoto et al. 2001). piD261/Bud32 also phosphorylates Ser-37 *in vitro* (Facchin, Lopreiato et al. 2003).

Like PRPK and piD261/Bud32 protein kinases, SsoPK5 was observed to autophosphorylate, specifically on threonine and serine residues. Several observations suggest that at least one of the residues modified by autophosphorylation is Thr-151. Threonine-151 lies between subdomains VII and VIII of SsoPK5, a region corresponding

to the activation loop that undergoes phosphorylation in most eukaryotic ePKs (Hanks and Hunter 1995; Huse and Kuriyan 2002). Second, the corresponding serine residue in piD261/Bud32 is known to be modified by autophosphorylation (Facchin, Lopreiato et al. 2002). Third, mutagenic alteration of Thr-151 to alanine or aspartate resulted in a marked decrease in autophosphorylation. Further, these mutationally-generated alterations impacted catalytic function. Replacement of Thr-151 by alanine (T151A) resulted in a protein with very low activity, suggesting that autophosohorylation at this site is necessary for optimum catalytic efficiency. This behavior mirrored that of altered forms of piD261/Bud32 in which autophosphorylated amino acid residues were replaced by alanines, which cannot be phosphorylated (Facchin, Lopreiato et al. 2002). The specific activity of an SsoPK5 variant with Thr-151 substituted by the potential phosphoryl mimic — aspartate (T151D) — was equal to that of the wild type enzyme. Taken together, the physical and functional characteristics of the mutagenically-altered forms of SsoPK5 indicate that Thr-151 is a site of autophosphorylation, and that autophosphorylation of this residue enhances the catalytic activity of the enzyme.

<u>By what mechanism does ADP-ribose enhance the activity of SsoPK5?</u>

The nucleotides ADP-ribose or 5'-AMP both exerted salutary effects on the activity of SsoPK5. However, the mechanism by which they do so appears to be complex. Based on the observations reported herein, we postulate that ADP-ribose acts as a pseudochaperone, facilitating a conformation transition of SsoPK5 to a fully catalytically competent state.

SsoPK5 activation required incubation with ADP-ribose and 5'-AMP at least 30 min at 65°C. Moreover, activation proved surprisingly powerful and specific. ADP-ribose activated SsoPK5 at concentrations as low as 10 nM. In addition, neither 5'-ADP, NAD (H), or cyclic ADP-ribose — each of which share important structural features with ADP-ribose — exhibited any capacity to enhance the catalytic activity of SsoPK5.

Although inclusion of ATP during pre-incubation had a beneficial effect, reducing variability between replicates, evidence shows that activation by nucleotides did not involve phosphotransfer to SsoPK5. First, little or no ^{32}P phosphate was incorporated into the enzyme when it was pre-incubated with [γ-^{32}P]ATP or when SsoPK5 was pre-incubated with ADP-ribose or 5'-AMP and [γ-^{32}P]ATP. Moreover, activation could be induced using the nonhydrolyzable ATP analogue AMP-PCP in the pre-incubation mixture with ADP-ribose or 5'-AMP. In addition, we observed SsoPK5 activation with the hydrolysis-resistant 5'-AMP analogue—AMPS.

Is ADP-ribose a physiologically-relevant effector?

In the *Eukarya,* poly(ADP-ribose) moieties are subject to rapid turnover that results in the production of monomeric ADP-ribose (Kraus and Lis 2003). While it is frequently speculated that ADP-ribose acts as an effector molecule, evidence supporting a signaling role for this nucleotide has proven elusive. A potential target for ADP-ribose are *macro* domain proteins, one of which, AF1521 from the thermophilic archaeon *Archaeoglobus fulgidus*, recently has been shown to bind ADP-ribose with high affinity

(Karras, Kustatscher et al. 2005). It has been reported that some proteins in *S. solfataricus* are modified by poly(ADP-ribosyl)ation (Faraone-Mennella, Gambacorta et al. 1998) and that its genome contains an ORF, *sso2899,* encoding a potential a *macro* domain protein. Moreover, it has been reported that *S. solfataricus* contains an archaeal poly (adenosine diphosphate (ADP)-ribose) polymerase (Castellano, Farina et al. 2009; Di Maro, De Maio et al. 2009). It therefore appears possible, if not likely, that ADP-ribose is present and may act as an effector molecule in *A. fulgidus, S. solfataricus,* and other members of the *Archaea.* While SsoPK5 does not contain a *macro* domain, it may represent a novel target for this putative effector. Moreover, the association of poly(ADP-ribose)ylation- depoly(ADP-ribose)ylation in modulating gene transcription is the *Eukarya* (Kraus and Lis 2003) is consistent with the implication that PRPK (Abe, Matsumoto et al. 2001) and, presumably, other piD261/Bud32-family protein kinases also participate in transcriptional and telomerase regulation.

Another more speculative but interesting possibility is that the physiological effector for SsoPK5 is not ADP-ribose, but 2'-O-acetyl-ADP ribose. 2'-O-acetyl-ADP-ribose is produced by the Sir2 deacetylases, which catalyze the deacetylation of chromatin proteins in the *Eukarya* as well as several of the *Archaea,* including *S. solfataricus* using NAD^+ as cosubstrate (Bell and Jackson 2001; Bell, Botting et al. 2002). Deacetylation of lysine residues in chromatin proteins increases the latter's affinity for DNA and modulates, at least in part, the chromatin remodeling associated with transcriptional silencing. As is the case for ADP-ribose, it has long been speculated that 2'-O-acetyl-ADP-ribose is not merely a passive by-product of the Sir2 deacetylase, but an important effector molecule. This deduction has recently been reinforced by the

observations that the *macro* domain of histone H2A1.1, a product of alternative splicing, binds 2'-O-acetyl-ADP-ribose (Kustatscher, Hothorn et al. 2005). Moreover, 2'-O-acetyl-ADP-ribose along with the deacetylation of histones modulates the assembly of both heteroligomeric complexes of Sir deacetylases as well as complexes between Sir proteins and histone H4 (Liou, Tanny et al. 2005). This action is thought to be promoted by the binding of 2'-O-acetyl-ADP-ribose to one of the Sir proteins, possibly Sir3 (Liou, Tanny et al. 2005). A putative model 2'-O-acetyl-ADP-ribose binding to SsoPK5 is illustrated in Figure 5-1. As with ADP-ribose, the association of 2'-O-acetyl-ADP-ribose with proteins involved in modulating gene transcription is a nice connection in the cell.

Summary

SsoPK5 is an archaeal structural and functional member of the piD261/Bud32 protein kinase subfamily. Its DNA sequence, open reading frame (ORF) *sso0433*, exhibits 33% sequence identity to PRPK and 26% sequence identity to piD261/Bud32. Moreover SsoPK5, PRPK, and piD261 share many characteristics. SsoPK5 phosphorylates acidic proteins such as casein and RCML. It preferentially utilizes manganese as the metal cofactor, and like PRPK and piD261/Bud32, SsoPK5 can phosphorylate human p53 on Ser15.

PRPK must be pre-incubated with COS7 cell lysate; we show that for SsoPK5 to possess optimal phosphotransferase activity it too must be pre-incubated with specific molecules. Specifically, SsoPK5 phosphotransferase activity is stimulated by genomic

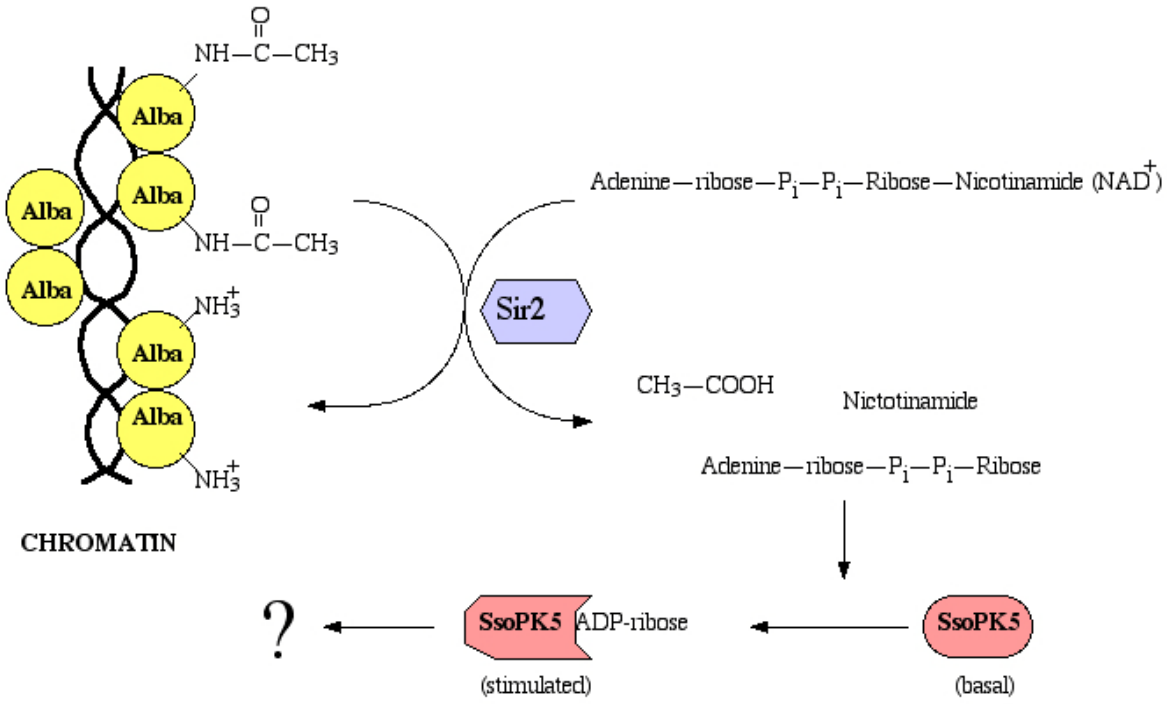

Figure 5-1. 2'-O-acetyl-ADP-ribose is a catabolite from Sir2 deacetylases.

DNA isolated from salmon sperm and various archaeons as well as ADP-ribose and 5'-AMP. To achieve optimal phosphotransferase activity, SsoPK5 must be pre-incubated with ATP, AMP-PNP or AMP-PCP along with the activator, such as ADP-ribose or 5'-AMP. Interestingly, the γ-phosphate from ATP is not transferred to SsoPK5 nor is cleavage required during pre-incubation for SsoPK5 activation. Whether through transcriptional regulation models or as a signaling molecule both ADP-ribose and 5'-AMP show interesting promise for physiological activators of SsoPK5. In order to elucidate SsoPK5 specific role in *S. solfataricus*, endogenous substrates must be identified. One potential approach for so doing would be to overexpress SsoPK5 in *S. solfataricus* cultured in the presence of radiolabelled inorganic phosphate. Potential phosphoacceptor proteins should exhibit an increased level of phosphorylation when compared to the control samples. Another point of investigation is the apparent dependence of SsoPK5 autophosphorylation on the presence of a phosphorylated substrate. Is this a hieracheal phosphorylation event?

Particular care should be taken when purifying SsoPK5. Even at room temperature, SsoPK5 phosphotransferase activity can disappear in a short period of time (within two weeks). Therefore, it is essential to have a continuous supply of transformed BL21 (DE3) RIL *E. coli* cells on hand. So, glycerol permanents were made prior to induction of the large-scale culture. Also, when assaying SsoPK5 for phosphotranferase activity, SsoPK5 must be pre-incubated with ATP or ATP-analogs and/or activators at 65°C for no less than 30 min. SsoPK5 must be added to the pre-incubation mixture last.

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