

**PURIFICATION AND CHARACTERIZATION OF A PROTEIN  
PHOSPHATASE (PP1-ARCH) FROM THE ARCHAEOBACTERIUM  
*SULFOLOBUS SOLFATARICUS*, ISOLATION AND EXPRESSION  
OF ITS GENE**

**By**

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(ABSTRACT)**

PP1-Arch was verified as a protein phosphatase by both acid molybdate extraction and thin layer electrophoresis. Soluble fraction was prepared from *Sulfolobus solfataricus*, from which PP1-Arch was purified over 1000-fold by DE-52 ion-exchange, hydroxyapatite, gel filtration (G-100), and Mono Q FPLC chromatography. PP1-Arch was identified from the final purified sample by renaturation on an SDS-polyacrylamide gel. The molecular size of PP1-Arch was determined by both gel filtration chromatography and SDS-PAGE as 28 kDa and 33 kDa, respectively, which suggests that PP1-Arch is a monomer. PP1-Arch was found stable at temperatures as high as 90°C. Activation constants for the divalent metal ions  $Mn^{2+}$  and  $Ni^{2+}$ , and the  $K_m$  for phosphocasein were determined. Myosin light chain was found to be a substrate for PP1-Arch *in vitro*. EDTA,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $P_i$ , and  $PP_i$  were shown to be inhibitors of PP1-Arch, while many

compounds known to affect eukaryotic protein phosphatase activities were found to be without noticeable effect.

N-terminal and an internal peptide sequence of the enzyme were obtained. The gene for PP1-Arch was cloned by a combination of "touchdown" PCR and conventional cloning techniques. The PP1-Arch gene was sequenced on both strands, and the sequence was compared with ones from eukaryotes and bacteriophage  $\lambda$ . The sequence homology between PP1-Arch and PP1/PP2A/PP2B suggests that they belongs to the same genetic family.

A recombinant plasmid which was derived from pT7-7 was constructed for expression of PP1-Arch. The PP1-Arch gene was expressed in *E.coli*. and the activity of the expressed enzyme was tested and shown to be divalent metal ion-dependent. Formation of inclusion bodies of expressed PP1-Arch was demonstrated.

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## LIST OF ABBREVIATIONS

|       |                                                          |
|-------|----------------------------------------------------------|
| Amp   | ampicillin                                               |
| ATP   | adenosine triphosphate                                   |
| bp    | base pair(s)                                             |
| BSA   | bovine serum albumin                                     |
| cAMP  | 3', 5'-cyclic adenosine monophosphate                    |
| CAPS  | 3-[cyclohexylamino]-1-propane sulfonic acid              |
| cGMP  | 3', 5'-cyclic guanosine monophosphate                    |
| CIAP  | calf intestinal alkaline phosphatase                     |
| DEPC  | diethyl pyrocarbonate                                    |
| dNTP  | deoxynucleoside triphosphate                             |
| DTT   | dithiothreitol                                           |
| EDTA  | ethylenediaminetetraacetic acid                          |
| EGTA  | ethylenedioxydiethylenedinitrolo tetraacetic acid        |
| EtBr  | ethidium bromide                                         |
| FPLC  | fast protein liquid chromatography                       |
| IPTG  | isopropylthio- $\beta$ -D-galactopyranoside              |
| kDa   | kilodaltons                                              |
| LB    | Luria Broth                                              |
| MES   | 2-[N-morpholino]ethanesulfonic acid                      |
| PAGE  | polyacrylamide gel electrophoresis                       |
| PCR   | polymerase chain reaction                                |
| PMSF  | phenylmethanesulphonyl fluoride                          |
| PP    | serine/threonine protein phosphatase                     |
| PVDF  | polyvinylidene difluoride                                |
| Q-    | quaternary ammonium-                                     |
| SDS   | sodium dodecyl sulfate                                   |
| TCA   | trichloroacetic acid                                     |
| TLC   | thin layer chromatography                                |
| TLCK  | tosyl-L-lysine chloromethyl ketone                       |
| TMAC  | tetramethylammonium chloride                             |
| TPCK  | tosyl-L-phenylalanine chloromethyl ketone                |
| Tris  | tris(hydroxymethyl)aminomethane                          |
| X-gal | 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside |

## INTRODUCTION

### **Protein phosphorylation in eukaryotes and eubacteria**

Protein phosphorylation is a reversible, covalent protein modification process that was first identified from the study of glycogen metabolism in skeletal muscle by E. G. Krebs and E. H. Fisher in 1956. Protein phosphorylation has been shown to play important roles in the regulation of a variety of cellular events (Fisher and Krebs, 1990; Cohen, 1989; Ingebritsen *et al.*, 1983; Cohen, 1992), including glycogen metabolism, muscle contraction, signal transduction, fatty acid and sterol biosynthesis, gene transcription and translation, cell differentiation and proliferation, and the transport of ions and metabolites across membranes.

Protein phosphorylation and dephosphorylation is one of the major mechanisms for signal transduction in eukaryotic and eubacterial organisms (Cohen, 1992). Generally, extracellular agonists exert their effects on cells by activating or inhibiting transmembrane signalling systems that control the production of secondary messengers. The second messengers in turn bind to protein kinases and protein phosphatases and modulate their activities. Protein phosphorylation and dephosphorylation of substrate proteins triggers conformational changes that alter their functional properties, leading to physiological responses. In mammals, for instance, glycogenolysis is activated by  $\text{Ca}^{2+}$  during muscle contraction (Krebs and Beavo, 1979). The

effect of  $\text{Ca}^{2+}$  is to alter the activities of protein kinases and protein phosphatases, which shifts the balance of the activities of glycogen phosphorylase and glycogen synthase, and ultimately triggers glycogenolysis. In bacteria, a signal transduction system, the two component system, is important in chemotaxis (Stock *et al.*, 1992). The stimulus, generally some nutrient, binds to a receptor, inducing a conformational change of the receptor. This change causes the receptor to interact with a protein kinase, which in turn undergoes autophosphorylation of a histidine residue near its C-terminal end. That phosphoryl group is then transferred to an aspartate residue located near the N-terminal end of another protein, the response regulator. The phosphorylated response regulator binds to an output protein, such as a flagellar motor, which alters the cell's swimming pattern.

### **Classification and structure of protein kinases**

A protein phosphorylation network consists of protein kinases, protein phosphatases, and phosphoproteins. Protein kinases transfer the terminal phosphoryl group of a nucleoside triphosphate to an amino acid on the acceptor protein. In general, the preferred nucleoside triphosphate is adenosine triphosphate. Depending on the nature of amino acid to which the phosphoryl group is transferred, protein kinases can be classified as protein-serine/threonine kinases, protein-tyrosine kinases, protein-cysteine kinases and protein-aspartyl, -glutamyl or -histidyl kinases (Hunter, 1991). Many of those kinases are regulated by second messengers such as cAMP, cGMP,

Ca<sup>2+</sup>, or phospholipids (Fisher and Krebs, 1990).

Among the protein kinases, the protein-serine/threonine and protein-tyrosine kinases are the best characterized. Comparison of the primary structure of a large number of the eukaryotic protein-serine/threonine kinases and protein-tyrosine kinases revealed that they shared a homologous catalytic domain of about 270 amino acids (Hanks *et al.*, 1988). Those sequence alignments indicate that there are several invariable residues and highly conserved subdomains distributed through the catalytic domain.

## **Classification and structure of protein phosphatases**

### Classification of Protein Phosphatases

In contrast to protein kinases, protein phosphatases remove phosphoryl groups from phosphoproteins and release free inorganic phosphate. According to the nature of the amino acid upon which the enzyme acts, protein phosphatases can be classified into serine/threonine-specific (Cohen, 1991), tyrosine-specific (Tonks, 1990; Hunter, 1989), and other amino acid-specific protein phosphatases (Huang *et al.*, 1991). Some have dual specificities (Kim *et al.*, 1993; Potts *et al.*, 1993). Several years ago, P. Cohen introduced a classification scheme for the protein serine/threonine-specific protein phosphatases (Cohen, 1989). They were divided into two types according to the subunit of phosphorylase kinase that they dephosphorylated preferentially, and whether they were inhibited by two small heat and acid stable proteins (inhibitor 1 and inhibitor 2) (Huang and

Glinsmann, 1976). Type 1 serine/threonine specific protein phosphatases preferentially dephosphorylate the  $\beta$  subunit of phosphorylase kinase and are inhibited by inhibitor 1 or inhibitor 2. Type 2 serine/threonine specific protein phosphatases preferentially dephosphorylate the  $\alpha$  subunit of phosphorylase kinase and are insensitive to inhibitor 1 or inhibitor 2. The type 2 serine/threonine specific protein phosphatases can be subdivided into three groups based on their dependence on divalent cations: PP2A, PP2B and PP2C. Like PP1, PP2A has no absolute dependence on divalent cations for activity. PP2B and PP2C have an absolute requirement for  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , respectively.

#### Sequence analysis of PP1/PP2A/PP2B and PP2C

P. Cohen's classification of serine/threonine-specific protein phosphatases was based purely on substrate specificity, and activation or inhibition studies. It did not provide any clues concerning the structures of the enzymes or how they relate to one another. More recently, protein sequencing and cDNA cloning have been used to elucidate the structures of PP1, PP2A, PP2B, and PP2C. Comparison of the catalytic subunits of PP1 from diverse species revealed a very high degree of conservation among this class of protein phosphatases. Over 80% sequence identity was found among the enzymes ranging from fungi, to fruit flies, to humans (Cohen, 1988; Berndt *et al.*, 1987). The variable regions virtually were confined to the extreme  $\text{NH}_2$ - or  $\text{COOH}$ -terminal regions of the enzymes. Likewise, the isoforms of PP2A share over 95% sequence identity (Stone *et al.*, 1987).

Somewhat surprisingly, PP1 and PP2A show  $\geq 40\%$  overall sequence identity between them (Cohen and Cohen, 1989). PP2B also showed sequence homology with PP1/PP2A, but to a lesser extent (Kincaid *et al.*, 1990). High sequence identity exists among the isoforms of PP2C (McGowan and Cohen, 1987; Arino *et al.*, 1988), but not between PP2C and PP1/PP2A/PP2B. Therefore, on a molecular basis, the serine/threonine-specific protein phosphatases comprise two distinct gene families. PP1, PP2A, and PP2B belong a single family, while PP2C belongs to a second, completely distinct gene family (Cohen and Cohen, 1989).

#### Type 1 protein phosphatases

A number of different heteromeric forms of PP1 have been characterized. They associate with different regulatory subunits that modulate their activity or subcellular localization (Hubbard and Cohen, 1993; Cohen, 1989). In extracts from mammalian tissues, the majority of PP1 was associated with other cellular components, such as glycogen (Ingebritsen *et al.*, 1983) or myosin (Chisholm and Cohen, 1984). This is the results of its association with the so-called G and M subunits that anchor it to glycogen particles or myofibrils, respectively.

Among the different forms of PP1, the ATP-Mg<sup>2+</sup>-dependent protein phosphatase has been the most extensively studied (Hemmings *et al.*, 1982). The catalytic subunit is bound to a regulatory subunit that was originally named inhibitor 2. Normally the holoenzyme is inactive. In this state, inhibitor 2 has been phosphorylated by the cAMP-dependent protein kinase.

However, dephosphorylation of inhibitor 2 triggers a conformational change in the catalytic subunit that causes it to become fully active.

Considering that PP1 is highly conserved through evolution, PP1 must play important roles inside the cell. For example, development of insulin-dependent diabetes has been shown to correlate with the reduction in the level of PP1 (Bollen and Stalmans, 1984). PP1 is also involved in cell cycle events. In yeast, the product of the *bws1+* gene, which shares a 82% sequence identity with the catalytic subunit of mammalian PP1, was shown to be an inhibitor of cell division when it was overexpressed (Booher and Beach, 1989; Cyert and Thorner, 1989).

### Type 2 protein phosphatases

The isoforms of PP2A contain a catalytic subunit C (35-40 kDa), a structural subunit (A), and a regulatory subunit (B). The B subunit modulates the substrate specificity of PP2A, and the association of B subunit with the AC core attenuates the activity of PP2A (Hendrix *et al.*, 1993; Kamibayashi *et al.*, 1994). PP2A can be activated by polycations such as polylysine, or histones, and this activation phenomenon is often used as an identifying characteristic of PP2A (Ingebritsen and Cohen, 1983). PP2A has been shown to play a role in the regulation of muscle contraction (Bialojan *et al.*, 1987),  $\text{Ca}^{2+}$  transport (Hescheler *et al.*, 1988), protein synthesis (Pato *et al.*, 1983), and cell division and growth (Sontag *et al.*, 1993).

PP2B, a  $\text{Ca}^{2+}$ /calmodulin-regulated protein phosphatase, is a heterodimer consisting of a catalytic subunit and a calmodulin-like  $\text{Ca}^{2+}$ -

binding subunit (Klee *et al.*, 1979). In humans, it was observed that PP2B has its highest concentration inside neurons. Therefore, it sometimes is called calciuneurin. PP2B is believed to be involved in the regulation of ion channels in mammalian tissues (Armstrong, 1989).

PP2C can dephosphorylate a broad range of protein substrates *in vitro*. Its activity is  $Mg^{2+}$ -dependent (McGowan and Cohen, 1988; Tsuiki *et al.*, 1988). However, this probably is not of regulatory significance. The physiological role of PP2C has yet to be elucidated.

Within the two gene families of eukaryotic serine/threonine-specific protein phosphatases, PP1/PP2A/PP2B and PP2C, the extremely high degree sequence identity within each family makes it difficult to identify the functionally important residues involved in catalysis, substrate binding, and other functions. In order to identify these 'hotspots' among eukaryotic protein phosphatases, the sequence of a related enzyme from a more evolutionarily divergent organism is required. In an effort to address this issue, we decided to study the protein phosphatases of the archaeobacteria.

## **Archaeobacteria**

For many years living organisms were classified into two main phylogenetic kingdoms, prokaryotes, unicellular organisms lacking nuclei, and eukaryotes. The existence of the archaeobacteria originally was proposed in 1977 on the basis of major differences in the catalogs of archaeobacterial and eubacterial 16S rRNAs (Woese and Fox, 1977). During the past decade,

the accumulation of large quantities of gene sequences from a variety of different organisms has led to the demise of the two kingdom classification system. Using 16S rRNA as a molecular chronometer (Olsen *et al.*, 1985; Olsen and Woese, 1994), C. R. Woese suggested that there are at least three distinct kingdoms of living organisms, the eubacteria, the eukaryotes and the archaeobacteria (Woese *et al.*, 1990).

### Classification and unique biochemical features of archaeobacteria

Within the archaeobacterial kingdom, there are three groups: methanogens, halophiles and sulphur-dependent archaeobacteria (Jones *et al.*, 1987). The methanogens are anaerobes that reduce carbon dioxide to methane, the halophiles grow optimally at high concentrations of salt, and the sulphur-dependent archaeobacteria, which generally are thermophiles, generate energy via oxidation of molecular sulphur.

Archaeobacteria have a number of unique biochemical features. Instead of the straight-chain fatty acyl ester-linked glycerolipids with an sn-1,2-glycerol configuration found in eubacterial and eukaryotic membranes, archaeobacterial lipids consist of isoprenyl ether-linked glycerolipids with a sn-2,3-glycerol configuration (Kates and Kushwaha, 1988). The presence of tetraethers allows the formation of rigid lipid monolayer instead of the usual bilayer. Having ether-linked lipids may offer a selective advantage for the archaeobacteria, which often inhabit hostile environments, since ethers are very resistant to the acid/base hydrolysis. Cell envelopes of the archaeobacteria are different distinctly from those of eubacteria (Kandler and

Konig, 1993). Murein, the membrane characteristic of gram-negative bacteria, is not found in the archaeobacteria.

With regard to glucose catabolism, the Entner-Doudoroff pathway is utilized by some eubacterial organisms. However, in the archaeobacteria, glucose catabolism has been shown to proceed via a modified Entner-Doudoroff pathway (Danson, 1993). The sulphur-dependent archaeobacteria have a non-phosphorylated Entner-Doudoroff pathway, one in which the 2-keto-3-deoxygluconate undergoes direct aldol cleavage to pyruvate and glyceraldehyde, that is, without any prior phosphorylation.

#### Evolutionary relationships among eubacteria, archaeobacteria and eukaryotes

Morphologically, archaeobacteria are distinctly prokaryotic, like the eubacteria. They are unicellular organisms lacking a nuclei, and have a single RNA polymerase. However, archaeobacteria have many typically eukaryotic characteristics (Rivera and Lake, 1992; Woese, 1994). Archaeobacterial DNA-directed RNA polymerases more closely resemble those of eukaryotes than those of eubacteria (Zillig *et al.*, 1980). Some mRNAs have long polyadenylated sequences at their 3'-termini (Brown and Reeve, 1986). TATA box homologs were also found in archaeobacterial promoter elements (Thomm and Wich, 1988). Basic histone-like proteins associated with DNA has been found in archaeobacteria (Brown *et al.*, 1989). Unlike eubacteria, archaeobacteria use methionyl-tRNA, as do eukaryotes, instead of N-formylmethionyl-tRNA for the initiation of protein synthesis (Gupta, 1985). Archaeobacteria contain an elongation factor-2 (EF-2) that is

ADP-ribosylated by diphtheria toxin (Schroder and Klink, 1991), as are eukaryotic EF-2s. Many archaeobacterial ribosomal proteins more closely resemble those of eukaryotes than those of eubacteria (Ramirez *et al.*, 1989; Shimmin *et al.*, 1989).

All these genetic and biochemical features support the notion that, although distinct, archaeobacteria are more closely related to eukaryotes than are eubacteria. Presently, it is believed that there was a common ancestor that eventually gave rise all three classes of organisms. This ancestor evolved into eubacteria in one direction, and in the other direction, into archaeobacteria first, then into eukaryotes (Woese, 1994) (Figure 1).

#### *Sulfolobus solfataricus*: biochemical and genetic features

Among all species of the archaeobacteria, *Sulfolobus*, which belongs to the sulphur-dependent archaeobacteria, represents the genus with the most consistently eukaryotic features with regard to the structure of their 5S rRNA, the shape of 50S ribosomal subunit, etc. (Brown *et al.*, 1989). In fact, in a two domain phylogenetic tree, the eocytic tree, *Sulfolobus solfataricus* is group with eukaryotes (Lake *et al.*, 1984). *Sulfolobus* was first isolated from a thermal acid hot spring in Yellowstone National Park by Brierley in 1966 (Brierley *et al.*, 1973), and has since been found in hot springs which located throughout the world. All these springs were characterized by low pH (less than 3.0) and high temperature (65<sup>0</sup>C -90<sup>0</sup>C).

Among the *Sulfolobus* genus, *Sulfolobus solfataricus* was first isolated and identified by Brock *et al.* in 1972 (Brock *et al.*, 1972). Morphologically,

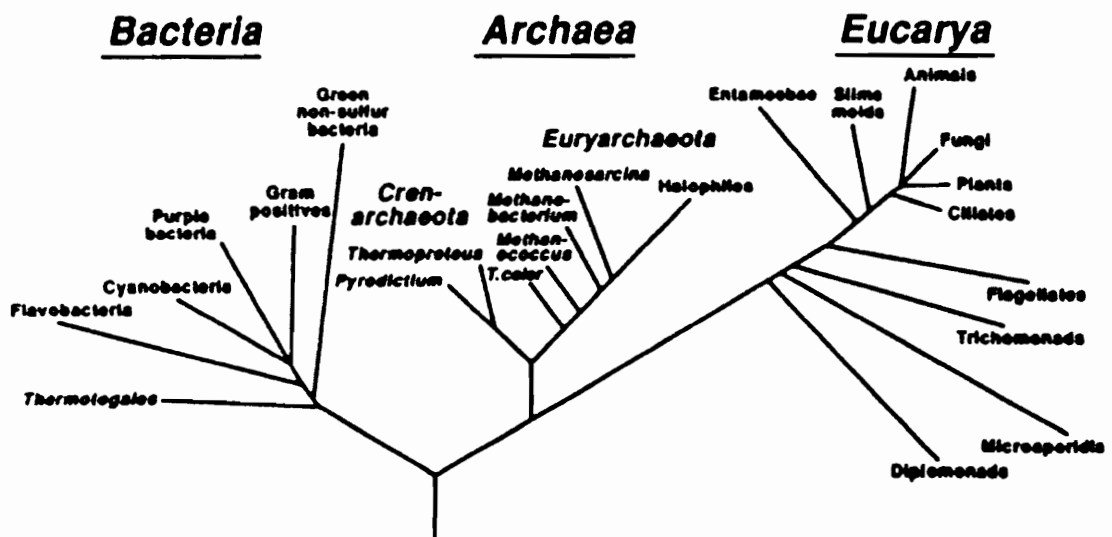


**Figure 1. Universal phylogenetic tree in rooted form, showing the three domains, Archaea (Archaeobacteria), Bacteria and Eucarya (Eukaryote). Adapted from C. R. Woese (1994).**

*Sulfolobus solfataricus* is a spherical organism. It can grow autotrophically on sulfur and heterotrophically on yeast extract. Although *Sulfolobus solfataricus* grows in extremely acidic environments, it maintains a neutral cytoplasmic pH (Schafer *et al.*, 1990). *Sulfolobus solfataricus* has a genome whose size is about 3000 kb, which is in the same range as a typical eubacterial chromosome. The GC content of its genome is about 36% (Brown *et al.*, 1989). No direct relationship between its high growth temperature and its G+C content has been discovered (Brown *et al.*, 1989). A consensus promoter motif (TTTAA/TA) and a T-rich sequence that is probably required for the termination process have been found in many *Sulfolobus* genes (Brown *et al.*, 1989). It is not yet known if the replication of *Sulfolobus solfataricus* chromosome starts at a single origin, as in eubacteria, or at multiple origins, as in eukaryotes. So far, the archaeobacteria have been shown to employ the universal genetic code (Brown *et al.*, 1989). Studies *in vitro* have shown that the binding of histone-like proteins to *Sulfolobus solfataricus* dsDNA increases the  $T_m$  of linear dsDNA from 25°C to 40°C higher over that of dsDNA itself (Grote *et al.*, 1986), which suggests that these histone-like proteins play a major role in stabilizing DNA in the thermophilic archaeobacteria.

### Protein phosphorylation in archaeobacteria

Protein phosphorylation was first discovered in eukaryotes, and for many years it was considered to be restricted to them. Later, it was found that protein phosphorylation exists in eubacteria and archaeobacteria also. In

*Sulfolobus acidocaldarius*, Skorko (1984) demonstrated that when the cells were grown in the presence of [<sup>32</sup>P]phosphate, four phosphoproteins could be detected on SDS-PAGE. Phosphorylation of one of those proteins was only observed in the late growth phase, suggesting that the activity or expression of one or more protein kinases, or expression of the phosphoprotein itself, was dependent on growth stage. In addition to ATP, Skorko (1989) suggested that polyphosphate could also serve as a source of phosphoryl groups for protein kinases in archaeobacteria. In *Halobacterium halobium*, the phosphorylation of some proteins was found to be light-dependent in a process that required a rhodopsin-like protein, and more recently a Mg<sup>2+</sup>-activated p-NPPase also has been found in this organism (Spudich and Spudich, 1981; Bonet *et al.*, 1993). In our laboratory, J. S. Cantwell was first to demonstrate the presence of divalent metal ion-dependent protein phosphatase activity in *Sulfolobus solfataricus* (Kennelly *et al.*, 1993). Later, this enzyme was named PP1-Arch. The studies of Oxenrider and Kennelly (Oxenrider and Kennelly, 1993; Oxenrider *et al.*, 1993) demonstrated that a similar protein phosphatase activity also existed in a methanogen and a halophile. All of these results indicate that protein phosphorylation is a universal phenomenon among living organisms (Kennelly and Potts, 1994).

### **Specific aims and significance of this project**

Since *Sulfolobus solfataricus*, an archaeobacterium, is thought to be evolutionarily related, but divergent from eukaryotes, we hypothesized that by obtaining the sequence of PP1-Arch from *Sulfolobus solfataricus* and

comparing it with those from eukaryotic organisms, we could be able to identify and study the important functionally important residues in those eukaryotic protein phosphatases. Such a study will also help us to better understand the protein phosphorylation network in the archaeobacteria.

#### Specific aims of this project

1. To verify that PP1-Arch is a protein phosphatase.
2. To purify PP1-Arch from *Sulfolobus solfataricus*.
3. To characterize PP1-Arch and obtain its partial amino acid sequence.
4. To clone and sequence the gene for PP1-Arch and compare its DNA-derived amino acid sequence with those of protein phosphatases from eukaryotic and eubacterial organisms.
5. To express PP1-Arch in *E.coli*.

## MATERIALS

All chemicals were purchased from Fisher or Sigma and were of analytical or HPLC grade. Electrophoresis apparatus and reagents were purchased from BioRad.  $^{32}\text{Pi}$ ,  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ,  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ ,  $[\alpha\text{-}^{35}\text{S}]\text{dATP}$  were from Du Pont-New England Nuclear. Eco-Lume liquid scintillation mixture was from ICN. Sephadex G-25 and G-100 were from Sigma. Mono Q was from Pharmacia. CM-Trisacryl was from Sepracor. DE-52 cellulose was from Whatman. Hydroxyapatite was from Bio-Rad. Cellulose thin-layer plates were from EM Science. Okadaic acid was from LC Services Corporation. Restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase (CIAP), T4 polynucleotide kinase, Proteinase K, pGEM-3Z, pGEM-T, LambdaGEM-11, X-gal, IPTG, Magic PCR miniprep, Magic plasmid miniprep and Magic clean-up system are from Promega. DNA Sequenase<sup>TM</sup> kit was from United States Biochemical Corporation. Taq polymerase PCR kit was from Perkin Elmer Cetus. Vent DNA polymerase is from New England Biolabs. X-ray films were from Fisher or Eastman Kodak Company.

## GENERAL PROCEDURES

All procedures were performed at room temperature unless otherwise specified. Distilled and deionized  $\text{H}_2\text{O}$  (dd $\text{H}_2\text{O}$ ) was used for making all the solutions. When experiments were performed at  $4^\circ\text{C}$ , all solutions were

prechilled to 4°C before they were used.

**List of solutions and media:**

|                           |                                                                                                                                                                                                                     |
|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 4 x SDS sample Buffer:    | 5%(w/v) SDS, 40%(v/v) glycerol, 0.05%(w/v) bromophenol blue.                                                                                                                                                        |
| 50 x Denhardt's solution: | 1%(w/v) ficoll (Type 400, Pharmacia), 1%(w/v) polyvinylpyrrolidone, 1%(w/v) BSA (Fraction V, Sigma).                                                                                                                |
| 20 x SSC:                 | 3 M NaCl, 0.3 M sodium citrate, pH 7.0                                                                                                                                                                              |
| TBE:                      | 50 mM Tris base, 50 mM Boric acid, 10 mM EDTA, pH 8.0                                                                                                                                                               |
| LB:                       | per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH to 7.0 with NaOH                                                                                                                                         |
| M9:                       | per liter: 6 g Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O, 3 g KH <sub>2</sub> PO <sub>4</sub> , 0.5 g NaCl, 1 g NH <sub>4</sub> Cl, 2 mM MgSO <sub>4</sub> , 0.2 mM CaCl <sub>2</sub> , 20%(w/v) Maltose. |

**Spectral and pH measurements:** Ultraviolet and visible absorbance measurements were performed on a Hitachi UV-2000 Spectrophotometer. All pHs were measured on a Accumet Model 10 pH meter (Fisher).

**Conductivity measurement:** All conductivity measurements were performed at 4°C on a YSI Model 35 conductance meter (YSI).

**Radioactivity measurement:** All the radioactivity measurements were

performed on a Beckman LS5800 scintillation counter.

**Protein assays:** Protein determination was routinely performed according to Bradford (1976) using premixed reagent and a standardized solution of BSA from Pierce. The protein-dye sample absorbance was measured at 595 nm. BSA, 0 - 50  $\mu$ g, were used to generate a protein standard curve.

**SDS-PAGE:** SDS-polyacrylamide gels were prepared and run as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue as described by Fairbanks *et al.* (1971).

**Isoelectric focusing:** Isoelectric focusing was performed according to the protocol of Ausubel *et al.* (1987) except that urea was eliminated from the recipe. A minigel apparatus (Hoefer Scientific Instruments, Model 250) was used. Protein sample was mixed with 6 x sample buffer (50%(v/v) glycerol, 6%(v/v) Bio-Rad Ampholyte, pH 4-6) and loaded onto an isoelectric focusing gel. Electrophoresis was performed at 750 volts for 2 - 3 hours at 4°C. NaOH, 20 mM, was used as the upper tank solution and 20 mM acetic acid as the lower tank solution. Gels were stained with Coomassie Brilliant Blue as described by Fairbanks *et al.* (1971).

**Electroblotting proteins onto PVDF membranes and N-terminal amino acid sequence analysis:** The procedure for electroblotting proteins to PVDF membranes was adapted from LeGendre *et al.* (1993). Briefly, 20  $\mu$ g protein

phosphatase sample was subjected to SDS-PAGE on a 20% polyacrylamide gel. The gel was soaked in transfer buffer (10 mM CAPS, pH 11.0, containing 15%(v/v) methanol and 0.02%(w/v) SDS) for 20 minutes. Meanwhile, a PVDF membrane (Millipore) of the same size as the gel was immersed in methanol for 2-3 seconds, then rinsed with water for 2-3 seconds and soaked in transfer buffer for a minimum of 15 min. The gel and PVDF membrane were sandwiched between Whatman 3 mm filter papers, which were cut to match the size of the gel. The Whatman paper was soaked in transfer buffer prior to use. The transfer of proteins from the gel to the PVDF membrane was carried out using a semidry electroblotting apparatus (BioRad) run for 40 min at 12 volts. After the electrophoretic transfer, the PVDF membrane was stained for 5 min with 0.1%(w/v) Coomassie Brilliant Blue R-250 dissolved in 40%(v/v) methanol containing 1%(v/v) glacial acetic acid, then destained with several changes of 50%(v/v) methanol (Aebersold, 1989). Finally, the membrane was rinsed with several changes of water and dried in air. The region containing PP1-Arch was excised and subjected to direct N-terminal amino acid analysis, which was performed by S. Buckel at Pitman-Moore Co. (Terra Haute, IN).

**Molybdic acid extraction:** The measurement of inorganic phosphate by molybdate extraction was performed using a modification of the procedure of Martin and Doty (1949). Briefly, TCA-soluble supernatant, 60  $\mu$ l, from a standard protein phosphatase assay was placed in a 1.5 ml Eppendorf tube. Next, 200  $\mu$ l of molybdate reagent was added along with 10  $\mu$ l of 4 mM

K<sub>2</sub>HPO<sub>4</sub>. Molybdate reagent was made up of 1.5 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O and 1.4 ml of 18 M H<sub>2</sub>SO<sub>4</sub> made up to a final volume of 100 ml with water. Next, 200 µl of isobutanol:toluene (1:1, v/v) was added and the mixture shaken vigorously on a vortex mixer for 20 seconds, then centrifuged for 3 min at 12000 x g. An aliquot of the top (organic) layer, 50 µl, was removed and counted for <sup>32</sup>P radioactivity.

**Growth and storage of organisms:** *E.coli* strains were initially streaked on LB agar plates. A single colony was used to inoculate LB medium, 1 ml, containing appropriate antibiotic and grown overnight at 37°C with shaking. Aliquots of the overnight culture were stored at -70°C after adding sterile 80%(v/v) glycerol to a final concentration of 15%(v/v). The petri plates containing *E.coli* colonies were stored at 4°C.

The standard growth medium for *Sulfolobus solfataricus* [ATCC 35091] consisted of 26 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.6 g of KH<sub>2</sub>PO<sub>4</sub>, 2.44 g of MgSO<sub>4</sub>, 1.5 g of CaCl<sub>2</sub>·2H<sub>2</sub>O and 40 g of yeast extract in a final volume of 20 liters (de Rosa et al., 1975). The pH was adjusted to 3.0 with concentrated H<sub>2</sub>SO<sub>4</sub> (18 N). *Sulfolobus solfataricus* was grown in a continuous culture with vigorous aeration at 75°C. Media was pumped through at a rate of 20 liters per day. Gram staining was performed weekly to detect possible contamination of the culture. Harvested media was stored for 0-3 days at 4°C prior to harvesting. Cells were harvested three times per week. First, total volume of cell suspension was reduced from 40 or 60 liters to 5 liters using a Pellicon Cassette System membrane concentrator. The concentrated

cell suspension was then centrifuged for 30 min at 3500 x g. Cell pellets were stored frozen at -20°C until needed.

**Agarose and polyacrylamide gel electrophoresis of DNA samples:**

Samples of genomic or plasmid DNA were digested with restriction endonucleases in appropriate buffers as recommended by suppliers at 37°C from periods ranging from 30 min to overnight. Gels were prepared in TBE buffer with either low or high melting temperature agarose. The final agarose concentration was varied between 0.5 to 2.0%(w/v) depending on the size range of the DNA to be fractionated. Electrophoresis was performed at constant voltage (5V/cm) in TBE buffer containing 0.5 µg/ml EtBr. High melting temperature agarose gels were run at room temperature while low melting temperature agarose gels were run at 4°C.

DNA fragments too small to be fractionated on agarose (<0.5kb) were fractionated using 5% - 10% polyacrylamide gels in 1 x TBE buffer (Sambrook *et al.*, 1989).

**Dephosphorylation of DNA samples with CIAP:** All dephosphorylation reactions of DNA samples with CIAP were performed according to the protocol of supplier (Promega).

**Ligation and transformation:** Ligation of DNA fragments into plasmid vectors generally was performed in a final volume of 10 µl containing insert (400 ng), vector DNA (100 ng), 1 µl of 10x ligase buffer, 0.5 µl of 10 mM ATP, pH 7.0, and 3 units of T4 DNA ligase. The ligation mixture was

incubated overnight at 16°C. For DNA that had been digested with a single restriction enzyme, vector DNA was usually dephosphorylated with CIAP prior to use, which was performed according to the protocol of supplier.

DH5αF' cells were made competent by treatment with calcium chloride according to Silhavy *et al.* (1984), and stored at -70°C after adding sterile 80%(v/v) glycerol to a final concentration of 15%(v/v).

Transformation was performed by mixing 1 or 2 μl of ligation mixture with 100 μl of DH5αF' competent cells and incubating on ice for 30 min. Next, the mixture was heat shocked for 2 min by incubating at 42°C. Following this, LB medium, 1 ml, was added, and the sample was incubated for 1 hr at 37°C. The transformation mixture was then plated on a selective medium, such as LB<sup>Amp</sup>.

**Isolation of *Sulfolobus solfataricus* genomic DNA:** Genomic DNA from *Sulfolobus solfataricus* was isolated according to the protocol of Ausubel *et al.* (1987). Typically, *Sulfolobus solfataricus* cell pellet, 3 g, was suspended in 20 ml of TE buffer. SDS, 10%(w/v), and Proteinase K, 20 mg/ml, were added to a final concentration of 0.5%(w/v) and 100 μg/ml, respectively. The mixture was incubated for 1 hour at 37°C. Next, 2.5 ml of CTAB/NaCl solution containing 2%(w/v) cetyltrimethylammonium bromide (CTAB), 100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, and 6 M NaCl, was added to the mixture and incubated for 30 min at 65°C. The mixture was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and centrifuged for 10 min at 6000 x g. The DNA in the aqueous solution was precipitated by adding 2 volume of cold 95%(v/v) ethanol followed by

a centrifuging for 10 min at 6000 x g. The resulting DNA pellet was washed once in cold 70%(v/v) ethanol. The final DNA sample was dissolved in 10 ml of TE buffer, and the absorbance at OD 260 nm and OD 280 nm was measured to determine DNA concentration (Sambrook *et al.* 1989). The DNA solution was stored at 4°C.

**Labeling of oligonucleotides and random primer labeling of DNA with**

**<sup>32</sup>P:** <sup>32</sup>P labeling of oligonucleotides was performed as described by Ausubel *et al.* (1987). Typically, 75 pmol of oligonucleotide was used in each reaction. In addition to the oligonucleotide, the reaction mixture contained 75 pmol of [ $\gamma$ -<sup>32</sup>P]ATP (6  $\mu$ Ci/pmol), 7.5  $\mu$ l of 10 x T4 polynucleotide kinase buffer, and 30 units of T4 polynucleotide kinase. After incubation for 30 min at 37°C, the reaction was stopped by adding 2  $\mu$ l of 0.5 M EDTA, pH 8.0. The mixture then was applied to a Biospin 6 column (Bio-Rad) to separate the <sup>32</sup>P-labeled oligonucleotide from free ATP according to the protocol provided by supplier. Random primer labeling was performed according to the manufacturer's protocol using the Prime-a-gene<sup>TM</sup> labeling system (Promega) on 25 ng quantities of DNA. [ $\alpha$ -<sup>32</sup>P]dATP was chosen as the isotopically labeled deoxynucleotide.

**Southern Blotting:** Southern blotting was performed as described by Ausubel *et al.* (1987). *Sulfolobus solfataricus* genomic DNA, 10  $\mu$ g or 20  $\mu$ g, was digested sequentially with a number of individual restriction enzymes, then subjected to electrophoresis on a 1.0% agarose gel. Next, the

agarose gel was soaked for 30 min in 0.25 M HCl, 2 x 30 min in 1.5 M NaCl/0.5 M NaOH, and 2 x 30 min in 1.5 M NaCl/0.5 M Tris, pH 7.0, prior to blotting onto a nylon membrane in 20 x SSC. DNA transfer was performed via downward capillary transfer system (Ausubel *et al.*, 1987). The membrane was then baked for 30 min at 80 °C and the DNA on the membrane was UV-crosslinked by using an UV illuminator (Fisher) for 30 seconds at 254 nm. The membrane was then prehybridized at 37°C for 2 hours by gently shaking in a solution of 6 x SSC, 10 x Denhardt's solution (Denhardt, 1966), 0.5%(w/v) SDS, 50 mM sodium phosphate, pH 6.5, containing 125 µg/ml denatured salmon sperm DNA. Hybridization was performed by adding <sup>32</sup>P-labeled oligonucleotide probes to the above prehybridization solution and incubating overnight at 37°C. After hybridization, the membrane was rinsed once in washing solution (6 x SSC, 1%(w/v) SDS), then washed in the same solution for 30 min at room temperature. The membrane was then transferred to a prewarmed washing solution and washed for 15 min at 42°C. The membrane was subjected to autoradiography.

**Construction of *Sulfolobus solfataricus* genomic DNA library:** *Sulfolobus solfataricus* genomic DNA, 100 µg, was digested with *Eco*RI or *Hind*III. Then, the DNA was purified by Magic clean-up system (Promega) according to the manufacturer's protocols. The digested DNA was ligated into pGEM-3Z which had been digested with the same restriction enzyme and dephosphorylated with CIAP. The ligation mixture was transformed into

competent DH5 $\alpha$ F' cells to produce a DNA library. The library was plated onto LB agar containing ampicillin (100  $\mu$ g/ml), IPTG (40  $\mu$ g/ml) and X-gal (40  $\mu$ g/ml).

**Screening the genomic DNA library from *Sulfolobus solfataricus* with radiolabeled oligonucleotides and DNA fragments:** The preparation of nitrocellulose filters (MSI) containing the genomic DNA library of *Sulfolobus solfataricus* and hybridization of these filters were performed according to the protocols of Ausubel *et al.* (1987). When radiolabeled oligonucleotides were used as probes, the filters were hybridized in SSC hybridization solution (6 x SSC, 1 x Denhardt's solution, 0.05%(w/v) sodium pyrophosphate containing 100  $\mu$ g denatured salmon sperm DNA) for two days at 5 $^{\circ}$ C less than the calculated  $T_m$  value of the oligonucleotide probes, followed by washing in 6 x SSC, 0.05%(w/v) sodium pyrophosphate at 2 $^{\circ}$ C less than the calculated  $T_m$  value of the oligonucleotide probes. When DNA fragments were used as probes, hybridization was performed in 50%(v/v) formamide, 5 x SSC, 1x Denhart's solution, 10%(w/v) dextran sulfate, 0.1%(w/v) SDS and 10 mM Tris, pH 7.5 at 42 $^{\circ}$ C for overnight. High stringency washing was performed for 30 min at 70 $^{\circ}$ C in 0.2 x SSC containing 1%(w/v) SDS. Positive colonies were defined as those which visibly hybridized to probes on duplicate filters, as determined by autoradiography.

**DNA sequencing:** Double strand plasmids were used as templates for sequencing via the dideoxynucleotide chain termination method of Sanger *et*

*al.* (1977). Portions, 1 pmol, of universal primers T7 or SP6 (Promega) or synthetic oligonucleotides designed specifically for PP1-Arch gene (DNAgency, PA) were used as primers in the reaction (Table 1). When oligonucleotide 1 was used as primer, 16 pmol was used in the annealing reaction. The gene for PP1-Arch were sequenced on both strands. Sequencing reactions were performed following the protocol provided in the DNA Sequenase™ kit (USB). Reaction mixtures were subsequently analyzed by electrophoresis on gels containing 6%(w/v) acrylamide and 7 M urea in 1 x TBE buffer. Sequencing gels were run in 1 x TBE buffer for 1.5 hrs at a constant power rating of 60 watts. After electrophoresis, the gel was soaked for 10 min in 10%(v/v) glacial acetic acid, 12%(v/v) methanol, then dried under vacuum. Kodak BioMR films were used for autoradiography.

**Polymerase Chain Reaction:** DNA amplification was carried out using a GeneAmp PCR kit and a GeneAmp PCR system 9600 temperature cycler (Perkin Elmer Cetus) according to the manufacturer's instructions. Typically, in a volume of 100  $\mu$ l, either 1  $\mu$ g *Sulfolobus solfataricus* genomic DNA or 100 ng plasmid DNA was used as template. The reaction mixture also contained 100 pmol of each primer. The PCR cycles were as follows: the initial denaturation for 3 min at 94°C, following by 30 cycles at these three temperature settings: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 min. In the case of "touchdown" PCR (Roux, 1994), the following sequence was used. Each cycle involved denaturation for 1 min at 94 °C, followed by incubation for 1 min at the annealing temperature and

finally, incubation for 2 min at 72°C. For the first 45 cycles, the annealing temperature was decreased from 55°C to 41°C in 1°C steps, each step lasting 3 cycles. The last 10 cycles stayed at the annealing temperature of 40°C.

**Expression of PP1-Arch in *E.coli*:** The PP1-Arch gene was amplified by a PCR reaction in which 1 µg of *Sulfolobus solfataricus* genomic DNA was used as template and two oligonucleotide primers corresponding to the NH<sub>2</sub>-terminal sequence containing a *NdeI* site or COOH-terminal sequence containing a *SaII* site at its 5' end were chosen (Oligonucleotide 15 and 16, Table 1). Expression vector pT7-7 (Studier and Moffatt, 1986) and the PCR product of the amplified PP1-Arch gene were digested with both *NdeI* and *SaII*. After dephosphorylation of vector with CIAP, the vector and the digested PCR product were ligated and transformed into competent DH5aF' cells. The recombinant plasmid was isolated and transformed into *E.coli* strain BL21(DE3). A single colony was inoculated in 1 ml of M9<sup>Amp</sup> liquid medium (Sambrook *et al.*, 1989) and grown overnight at 37°C. A portion, 50 µl, of the culture was diluted with 2 ml of M9<sup>Amp</sup> medium and the culture grown at 37°C until an OD at 600 nm of 0.6-1.0 was reached. IPTG was added as a 200 mM stock solution in 10 µl to a final concentration of 1 mM. After two hours of induction, an aliquot of the culture, 100 µl, was centrifuged for 3 min at 12000 x g. The cell pellet was lysed in 2 x SDS sample buffer (2.5%(w/v) SDS, 20%(v/v) glycerol, 0.025%(w/v) bromophenol blue) and the total proteins were analyzed on a 12.5% SDS polyacrylamide gel. The remainder of cells were also collected by

**Table 1. Oligonucleotide sequences:** Directions (sense, 5' to 3'; antisense, 3' to 5') are given in parentheses. All oligonucleotide sequences were written 5' to 3' orientation. The first base of initiation codon for PP1-Arch gene is designated as + 1. Standard abbreviations to areas of degeneracy are the following: R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, H = A or C or T, B = G or T or C, V = G or C or A, D = G or A or T, N = A or C or G or T.

| Number                                                                                                          | Sequence                      | Position    |
|-----------------------------------------------------------------------------------------------------------------|-------------------------------|-------------|
| A. Oligonucleotides used in screening of <i>Sulfolobus solfataricus</i> genomic DNA library and "Touchdown" PCR |                               |             |
| 1 (sense)                                                                                                       | ATGAAYATWGARGARAC             | +1 - +17    |
| 2 (sense)                                                                                                       | ATGAAYATWGARGARACNTA          | +1 - +20    |
| 3 (sense)                                                                                                       | TTYGAYATWTTYGGNCARCA          | +40 - +59   |
| 4 (antisense)                                                                                                   | TCNGGRTAYTTNARYTTNGC          | +536 - +517 |
| B: Oligonucleotides used in DNA sequencing                                                                      |                               |             |
| 5 (sense)                                                                                                       | TAACTCTAAAATACCTTCAT          | -69 - -50   |
| 6 (sense)                                                                                                       | TATGTTGATAGGGAACCACA          | +187 - +206 |
| 7 (sense)                                                                                                       | ACAAACTTTCATTATGGATTTTTT      | +322 - +345 |
| 8 (sense)                                                                                                       | CTAGGGATCACGACAGTTGAAGAT      | +490 - +513 |
| 9 (sense)                                                                                                       | GAAGGAATATACTATTTTGGG         | +631 - +651 |
| 10 (sense)                                                                                                      | GTCAAAGATCAGGTATTTTATATC      | +811 - +834 |
| 11 (antisense)                                                                                                  | CTCTATTAATTTCTTTAATAT         | +258 - +238 |
| 12 (antisense)                                                                                                  | TATCCATTAAGTAGGACTGAG         | +443 - +423 |
| 13 (antisense)                                                                                                  | TCTAATACTGGGTAAGAAATC         | +624 - +604 |
| 14 (antisense)                                                                                                  | ACACTAAAATTATTCACGTCTTT       | +919 - +900 |
| C: Oligonucleotides used PCR amplification of PP1-Arch gene for expression : start and stop codons are in bold. |                               |             |
|                                                                                                                 | <i>NdeI</i>                   |             |
| 15 (sense)                                                                                                      | CCACAATCATATGAACATTGAAGAAACG  | -10 - +18   |
|                                                                                                                 | <i>SalI</i>                   |             |
| 16 (antisense)                                                                                                  | TTTTTGTCTGACTATACTATCTCTTCTAT | +892 - +865 |

centrifugation, and suspended in 50 mM MES, pH 6.5, containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 35  $\mu\text{g/ml}$  PMSF, and 5  $\mu\text{g/ml}$  leupeptin. The cell suspension was then subjected to either sonication for 1 min with output at 5, timer at 5, and power at 80 watts using a Model W185 sonicator (Heatsystems-Ultrasons, Inc.) or disrupted using a French Press at a pressure of 20000 pounds per square inch. The extract was then separated into soluble and insoluble fractions by centrifugation at 12000 x g for 10 min. The proteins of soluble supernatant fraction and insoluble pellet fraction were suspended in 2 x SDS sample buffer and analyzed by SDS-PAGE. The soluble fraction was also assayed for protein phosphatase activity.

## SPECIFIC PROCEDURES

**Preparation of  $^{32}\text{P}$ -labeled phosphocasein:**  $^{32}\text{P}$ -labeled casein was prepared as described by McGowan and Cohen (1989). Briefly, the following were combined in a 1.5 ml Eppendorf tube: 100  $\mu\text{l}$  of 5%(w/v) partially hydrolyzed casein solution (Sigma), 85  $\mu\text{l}$  of 50 mM Tris, pH 7.0, containing 60 mM  $\text{Mg}(\text{OAc})_2$ , and 1mM DTT; 10  $\mu\text{l}$  of 10 mM ATP, pH 7.5; and 40  $\mu\text{l}$  of 20  $\mu\text{Ci}/\mu\text{l}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP. Water was added to the mixture to a final volume at 350  $\mu\text{l}$ . A lyophilized preparation of the catalytic subunit of cAMP-dependent protein kinase (1000 units) (Sigma) was dissolved in 75  $\mu\text{l}$  of 50 mM Tris, pH 7.0, containing 1mM DTT, and transferred to the above Eppendorf tube. Another 75  $\mu\text{l}$  portion of this same buffer was then used to rinse the container. This was added to the mixture in the Eppendorf tube.

The final volume was 0.5 ml. The mixture was incubated for 8 hours at 30°C and the reaction was terminated by adding 50  $\mu$ l of 100 mM sodium pyrophosphate, pH 7.5, containing 100 mM EDTA.

A portion of the reaction mixture, 5  $\mu$ l, was taken and diluted into 1 ml of water, then 5  $\mu$ l of the diluted reaction mixture was counted for radioactivity in order to determine the specific radioactivity of the [ $\gamma$ -<sup>32</sup>P]ATP.

The rest of the reaction mixture was loaded onto a G-25 column (1.8 x 15 cm) which had been equilibrated in 50 mM Tris, pH 7.0, containing 5%(v/v) glycerol. The column was eluted with the same buffer and 1 ml fractions were collected. Portions, 5  $\mu$ l of each fraction, were counted for radioactivity. The first radioactive peak fractions were saved as <sup>32</sup>P-labeled phosphocasein and stored at -20°C until needed.

**Assay of phosphocasein phosphatase activity:** The phosphocasein phosphatase assay were performed by using a modification of the method of McGowan and Cohen (1988). Briefly, the following were added to an Eppendorf tube: 5  $\mu$ l of 50 mM MES, pH 6.5; 5  $\mu$ l of 50 mM MES, pH 6.5 containing 120 MnCl<sub>2</sub>; 5  $\mu$ l of 50 mM MES, pH 6.5 containing 2 mg/ml BSA; 5  $\mu$ l of [<sup>32</sup>P]phosphocasein solution (see "Preparation of <sup>32</sup>P-labeled phosphocasein") and 10  $\mu$ l of protein phosphatase sample. The final volume was 30  $\mu$ l.

Reactions were started by adding the [<sup>32</sup>P] phosphocasein solution. After mixing briefly on a vortex mixer, the reaction mixture was incubated for 60 minutes at 25°C. After this incubation, 100  $\mu$ l of 20%(w/v) TCA was

added to stop the reaction. The mixture was spun for 3 min at 12000 x g, and a 50  $\mu$ l aliquot of the TCA-soluble supernatant was removed and counted for  $^{32}\text{P}$  radioactivity.

**Renaturation of PP1-Arch:** Mono Q fraction, 15  $\mu$ l containing 7.5  $\mu$ g protein, was mixed with 5  $\mu$ l of 4 x SDS sample buffer and heated for 5 min at 65°C. The mixture was then applied to a 15% SDS polyacrylamide gel. After electrophoresis, the slab gel was soaked for 30 min in 50 mM MES, pH 6.5, containing 10 mM EDTA followed by 30 min in 50 mM MES, pH 6.5. Next, the gel was divided into 1.0 x 0.2 x 0.1 cm slices using a Bio-Rad Gel Slicer (Model 192). The slices were placed in individual Eppendorf tubes containing 30  $\mu$ l of 100 mM MES, pH 6.5, 0.66 mg/ml BSA, and either 40 mM  $\text{MnCl}_2$  or 10 mM EDTA and homogenized using a plastic pestle. After soaking overnight at 4°C, these homogenized gel slice mixtures were assayed for protein phosphatase activity by adding 10  $\mu$ l of  $^{32}\text{P}$ -phosphocasein, incubating for 2 hours at 45°C. The reaction was stopped by adding 200  $\mu$ l of 20%(w/v) TCA and working up as described previously.

**Partial digestion of PP1-Arch with V8 proteinase:** Mono Q fraction, 20  $\mu$ l containing 10  $\mu$ g protein, was applied to a 15% SDS-polyacrylamide gel and electrophoresed under standard conditions. The gel was stained with Commassie Brilliant Blue and the section containing PP1-Arch was excised. The gel slice was loaded at the top of another 20% SDS-polyacrylamide gel with high molarity buffer system for the separation of small proteins or peptides (Okajima *et al.*, 1993). The electrode buffer and the stacking gel

were the same as Laemmli's system (Laemmli, 1970). The separating gel was also the same except that the concentration of Tris was doubled. The sample loading buffer was 50 mM Tris, pH 6.8, containing 20%(v/v) glycerol, 0.1%(w/v) SDS, 0.2%(w/v) bromophenol blue, and 40 ng/ $\mu$ l V8 proteinase (Kennedy *et al.*, 1988). The protein from the gel slice was electrophoresed into the stacking gel until the dye front reached the interface of stacking gel and running gel. Electrophoresis was then stopped for two hours to allow proteolysis to proceed. After that, electrophoresis was continued as usual. The resulting peptides were then transferred to a PVDF membrane as described previously. The peptides on the PVDF membrane was visualized by staining with Commassie Brilliant Blue according to Aebersold (1989). Sections of the membrane containing two of the most prominent peptides were excised and subjected to amino acid sequence on an Applied Biosystem Model 477A gas-phase sequenator at Department of Biology at Virginia Tech (Blacksburg, VA).

## RESULTS

### Identification of TCA-soluble product as inorganic phosphate

When PP1-Arch was originally identified from *Sulfolobus solfataricus*, protein phosphatase activity was assayed by a method that employed TCA precipitation to separate product from substrates. Since the cleavage of phosphocasein by endogenous proteinases could generate small phosphopeptides that were TCA-soluble, it was possible that the radioactivity detected in TCA-soluble supernatant arose come from those phosphopeptides and not inorganic phosphate. Therefore, the identification of assay reaction product as inorganic phosphate became necessary in order to demonstrate that we indeed had identified a protein phosphatase.

#### Acid molybdate extraction

DE-52 Fraction, 5  $\mu$ l containing 5.5  $\mu$ g protein, was assayed for phosphocasein phosphatase activity using the standard TCA precipitation method described under "Specific Procedures". A portion, 30  $\mu$ l, of the TCA-soluble supernatant from the assay was removed and counted for [ $^{32}$ P] radioactivity. From the same TCA-soluble supernatant a 60  $\mu$ l portion was removed and analyzed by the molybdic acid extraction as described under "General Procedures". Table 2 shows the comparison of the amount of phosphate released determined by the standard TCA method and molybdic acid extraction. As both methods yielded comparable results, this indicated that the product of the reaction was inorganic phosphate and that we were

**Table 2. Identification of TCA soluble product as inorganic phosphate by acid molybdate extraction.** DE-52 fraction, 5.5  $\mu$ g, was assayed for phosphocasein phosphatase activity using the standard TCA method as described under "Specific procedures". A portion of the TCA soluble supernatant, 60  $\mu$ l, was removed and extracted for phosphate by acid molybdate as described under "General procedures". Shown is the net phosphate released during the assays as determined by each method.

| <u>Additions</u> |      |      | <u>Net Pi released during assay (pmol)</u> |                          |
|------------------|------|------|--------------------------------------------|--------------------------|
| DE-52 fraction   | Mn2+ | EDTA | TCA method                                 | Molybdic acid extraction |
| -                | +    | -    | 0.1                                        | <0.1                     |
| -                | -    | +    | 0.1                                        | <0.1                     |
| +                | +    | -    | 30.3                                       | 30.3                     |
| +                | -    | +    | 0.7                                        | 1.1                      |

therefore detecting a protein phosphatase.

### Thin Layer Electrophoresis

The identity of the reaction product was subsequently confirmed by thin layer electrophoresis. DE-52 fraction, 30  $\mu$ l containing 15  $\mu$ g protein, was assayed for phosphocasein phosphatase activity under standard conditions with the following exception: first, to minimize potential interference with thin layer electrophoresis, 0.2 mM  $\text{MnCl}_2$  was used in the assay reaction instead of 20 mM  $\text{MnCl}_2$ ; second, after incubation the reaction mixture was immediately subjected to centrifugal ultrafiltration on Centricon 10 for 15 minutes at 2500 x g instead of TCA precipitation to separate low molecular weight products, also to prevent interference with thin layer electrophoresis. Aliquots, 2  $\mu$ l each, of filtrate were applied to a cellulose thin-layer plate. M. Zylka performed the electrophoresis in 2.5%(v/v) formic acid, 7.8% glacial acetic acid, pH 1.9, for 2 hours at 250 volts according to the procedures described by Cooper *et al.* (1983). The identity of assay reaction product was verified as inorganic phosphate by comparison of its migration with that of a [ $^{32}\text{P}$ ]phosphate standard.

### **Purification of PP1-Arch from *Sulfolobus Solfataricus***

All the purification procedures were performed at 4 $^{\circ}$ C unless otherwise specified. Protein phosphatase activity was determined both in presence or absence of divalent metal ions in order to monitor its divalent ion-dependence. Protein concentrations were monitored by either measuring UV

absorbance at 280 nm or using the Bradford protein assay.

#### Preparation of soluble fraction of *Sulfolobus Solfataricus*

Sonication buffer: 20 mM MES, pH 6.5, 0.1 M NaCl, 1 mM EDTA, 1mM EGTA, 1mM DTT, 5  $\mu$ g/l leupeptin, 0.5 mM PMSF, 0.5 mM TLCK, 0.5 mM TPCK, 5  $\mu$ g/ml soybean trypsin inhibitor. DTT, PMSF, TPCK and TLCK were added on day of use. PMSF and TPCK were added as 100 x stock solutions in isopropanol and methanol, respectively.

The cell pellet, 200 g, was thawed and suspended in 5 volumes of cold sonication buffer. The sonicator (Model W185, Heatsystems-Ultrasons, Inc.) was set as follows: output at 8.5, timer at 10.5, and power at 130 watts. The cell suspension was kept on ice and sonicated for several periods of 1 min duration, with pauses of several minutes between each period to insure efficient cooling. After each sonication cycle, 1 ml of sample was taken and spun for 2 min at 12000 x g, and the OD 400 nm of the supernatant was then measured. When the OD 400 nm absorbance leveled off, it was considered that sonication was complete. The sonicated cell suspension was immediately centrifuged at 15000 x g for 30 min. The supernatant liquid, soluble fraction of *Sulfolobus solfataricus*, was used for the next purification step or stored at -20°C. Typically, the volume of soluble fraction from *Sulfolobus solfataricus* was 500 ml containing 1660 mg protein with a specific activity of 0.23 nmol Pi released/min/mg at 45°C.

### Batch absorption onto DE-52

The following procedure was performed at room temperature. Soluble fraction of *Sulfolobus solfataricus*, 500 ml, was passed through a prefilter of CM-Trisacryl (10 x 4 cm) onto a column of DE-52 (6.25 x 30 cm). Both columns were equilibrated with DE-52 buffer (10 mM MES, pH 6.5, 35  $\mu$ g/ml PMSF, 0.5 mM/ml EDTA, 0.5  $\mu$ g/ml leupeptin). The columns were washed with DE-52 buffer until there was no protein detectable in the flowthrough. The CM-Trisacryl prefilter was discarded and the DE-52 column was washed thoroughly with 150 mM NaCl in DE-52 buffer, then eluted with the same buffer, but the concentration of NaCl increased to 400 mM. The protein eluting with 400 mM NaCl was collected and pooled as DE-52 fraction I. Generally, DE-52 fraction I had a volume of 1000 ml containing 290 mg protein with a specific activity of 2.0 nmol Pi released/min/mg at 45°C.

### Second DE-52 Column: elution with a linear salt gradient

DE-52 fraction I was dialyzed overnight against DE-52 buffer, then applied to a second DE-52 column (2.5 x 40 cm). This column was washed in the same manner as the first column, then eluted with a salt gradient of 400 ml each of DE-52 buffer containing either 150 mM NaCl or 400 mM NaCl. Fractions, 10 ml each, were collected and assayed for activity (Figure 2). Active fractions were pooled as DE-52 fraction II. Typically, DE-52 fraction II had a volume of 260 ml containing 60 mg protein with a specific activity of 5.1 nmol Pi released/min/mg at 45°C.

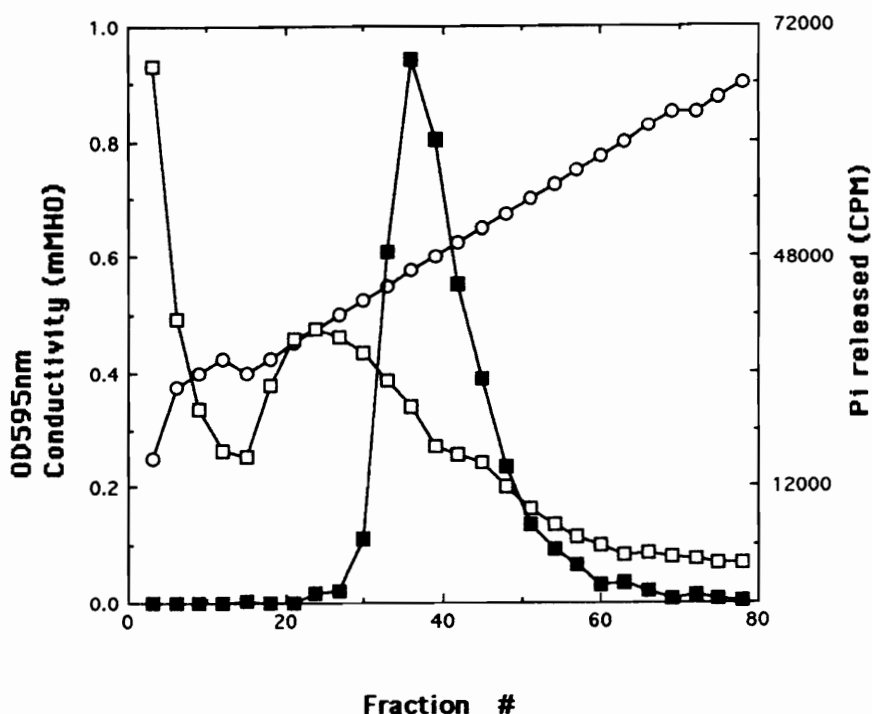


**Figure 2. The second DE-52 Column.** DE-52 fraction I, 1000 ml containing 290 mg protein, was applied to a DE-52 cellulose column (2.5 x 40 cm) that had been equilibrated in DE-52 buffer. The column was washed with the same buffer, then eluted with a salt gradient of 400 ml each of DE-52 buffer containing either 150 mM NaCl or 400 mM NaCl as described in the text. Aliquots, 20  $\mu$ l of each fraction, was measured for protein by Bradford assay. Shown is a plot of activity ( $\blacksquare$ ), protein concentration ( $\square$ ), and the conductivity following a 20-fold dilution ( $\circ$ ) as a function of fraction number.

### Hydroxyapatite Chromatography

DE-52 fraction II was dialyzed overnight against hydroxyapatite buffer (1 mM sodium phosphate, pH 6.5, containing 35 $\mu$ g/ml PMSF, 0.5 mM/ml EDTA and 0.5  $\mu$ g/ml leupeptin). Then, the dialyzed DE-52 fraction II was loaded onto a hydroxylapatite column (2.5 x 12 cm) equilibrated with hydroxyapatite buffer. The column was washed with the same buffer until no protein was detectable in the flowthrough. The phosphatase was eluted with a linear salt gradient consisting of 400 ml each of hydroxyapatite buffer containing either 1 mM sodium phosphate, pH 6.5 or 200 mM sodium phosphate, pH 6.5. Fractions, 10 ml each, were collected and assayed for protein phosphatase activity (Figure 3). Because phosphate was an inhibitor of PP1-Arch, the activity in Figure 3 can only be used to locate the active fractions, not as a quantitative measure. Active fractions were pooled as hydroxyapatite fraction. Typically, hydroxyapatite fraction had a volume of 228 ml containing 7.5 mg protein. It was then concentrated by centrifugal ultrafiltration at 3000 x g in a centriprep 10 to a volume of 1.9 ml.

### Gel filtration chromatography on Sephadex G-100

The concentrated hydroxyapatite fraction was applied to a column of Sephadex G-100 (5 x 100 cm) which had been equilibrated with G-100 buffer (20 mM MES, pH 6.5, containing 10 mM NaCl, 0.5 mM EDTA, 35  $\mu$ g/ml PMSF, 0.5  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml soybean trypsin inhibitor, and 5  $\mu$ g/ml benzamide). The column was eluted with the same buffer. Fractions, 2 ml, were collected and assayed for protein phosphatase activity (Figure 4, Panel A). Active fractions were pooled as G-100 fraction.

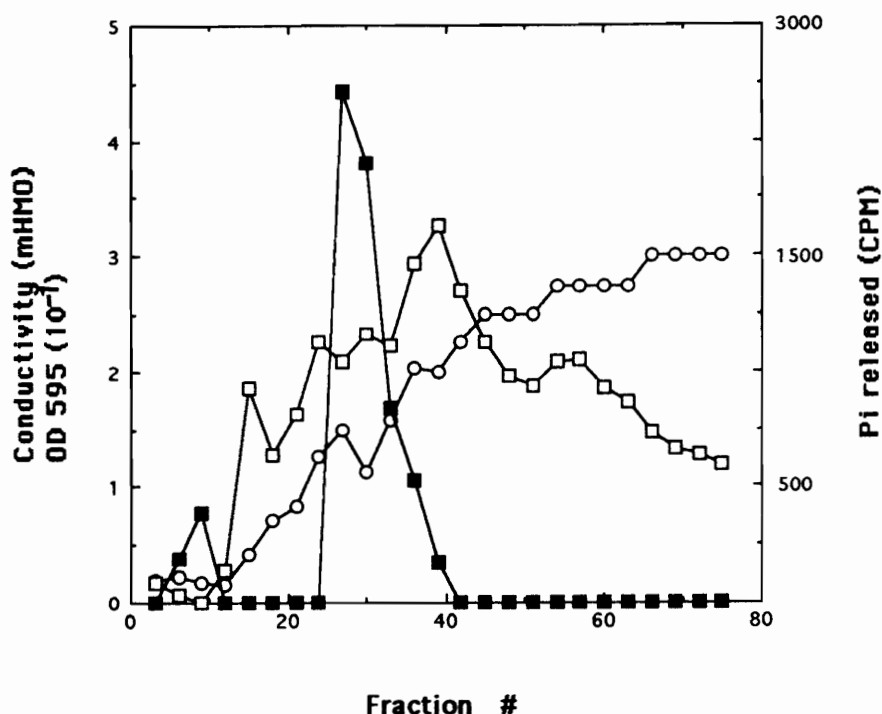
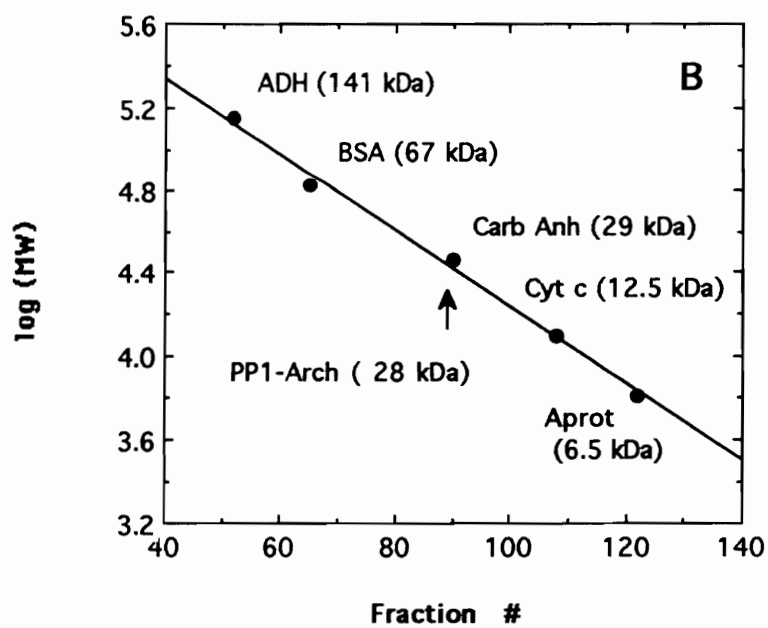
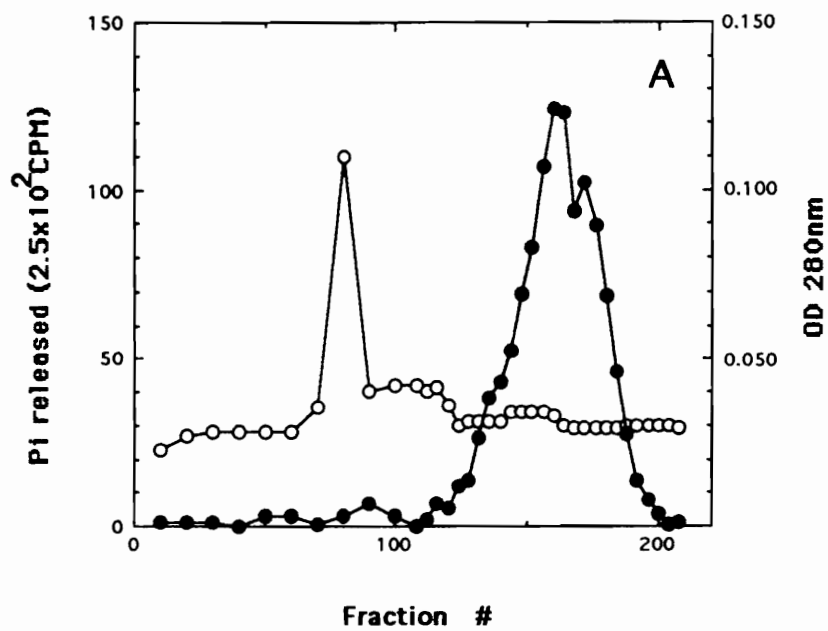


**Figure 3. Hydroxyapatite chromatography.** DE-52 fraction II, 260 ml containing 60 mg protein, was dialyzed against Hydroxyapatite buffer and applied to a 2.5 x 12 cm column of hydroxyapatite that had been equilibrated in hydroxylapatite buffer. After washing with the same buffer, the column was eluted with a linear salt gradient as described in the text. Aliquots, 100  $\mu$ l from each fraction, were measured for protein by Bradford assay. Portions of fractions, 10  $\mu$ l each, were assayed for phosphocasein activity under the standard conditions. Shown is a plot of activity(■), protein concentration(□) and conductivity(○) as a function of fraction number.

**Figure 4. Sephadex G-100 gel filtration chromatography.** Panel A: The concentrated hydroxyapatite fraction, 1.9 ml containing 7.5 mg protein, was applied to a column of Sephadex G-100 (5 x 100 cm). G-100 buffer was used to equilibrate and elute the column. Fractions, 2 ml each, were collected and protein was determined by measuring the absorbance of each fraction at OD280 nm. Portions, 10  $\mu$ l each, of fractions were assayed for phosphocasein phosphatase activity under the standard conditions. Shown is a plot of activity(●) and protein concentration(o) as a function of fraction number. Panel B: The concentrated DE-52 fraction II, 1 ml containing 2.3 mg protein, was applied to a column of Sephadex G-100 (1.5 x 100 cm) and eluted the same manner as in Panel A, except that fraction size was reduced to 1 ml. The column was calibrated using the following proteins: yeast alcohol dehydrogenase, Mr 141 kDa (ADH); bovine serum albumin, Mr 67 kDa (BSA); carbonic anhydrase, Mr 29000 (carb. Anh.); cytochrome c, Mr 12.5 kDa (Cyt c); and aprotinin, Mr 6.5 kDa (Aprot.). Shown is the elution position of PP1-Arch as compared to protein standards.



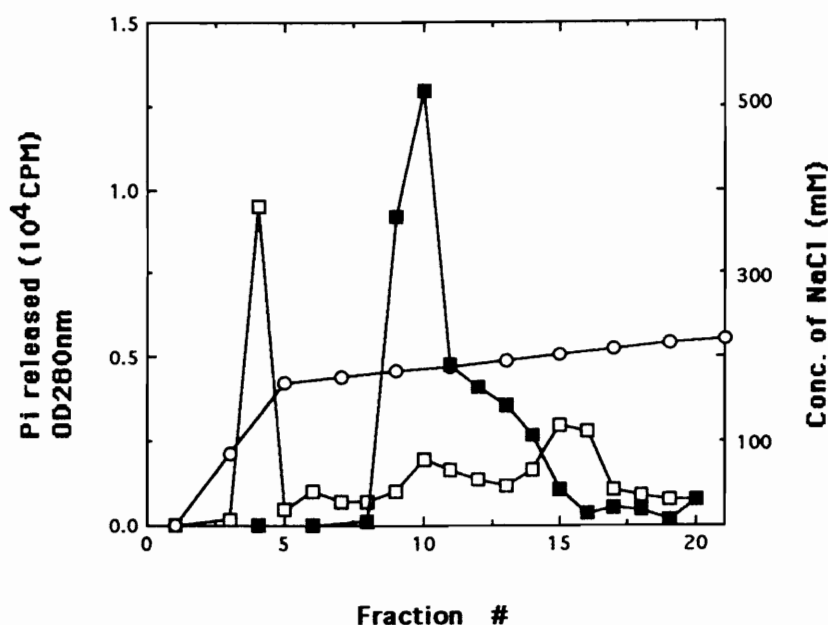


**Figure 5. Mono Q FPLC.** G-100 fraction was applied to a Mono Q-FPLC column (0.5 x 7 cm) which was equilibrated with Mono Q buffer. The column was washed with 5 ml Mono Q buffer, followed with a 2 ml salt gradient of 0 - 170 mM NaCl in Mono Q buffer. The protein phosphatase was eluted with a salt gradient (○) consisting of 17.5 ml each of Mono Q buffer containing either 170 mM NaCl or 270 mM NaCl as described in text. Fractions, 1 ml each, were collected and portions, 10  $\mu$ l each, were assayed for protein phosphatase activity (■). The elution of protein was monitored by OD280 nm (□).

**Table 3. Purification of PP1-Arch from *Sulfolobus solfataricus***

| <u>Fraction</u>         | <u>Protein</u><br>(mg) | <u>Activity</u><br>(nmol/min)                       | <u>Specific Activity</u><br>(nmol/min/mg) | <u>Recovery</u><br>(%) | <u>Enrichment</u><br>(fold) |
|-------------------------|------------------------|-----------------------------------------------------|-------------------------------------------|------------------------|-----------------------------|
| Crude Extract           | 1660                   | 380                                                 | 0.2                                       | 100                    | 1                           |
| DE-52 Fraction I        | 290                    | 580                                                 | 2.0                                       | 152                    | 10                          |
| Batch step              |                        |                                                     |                                           |                        |                             |
| DE-52 Fraction II       | 60                     | 304                                                 | 5.1                                       | 80                     | 26                          |
| Gradient elution        |                        |                                                     |                                           |                        |                             |
| Hydroxyapatite Fraction | 7.5                    | -did not measure due to inhibition by Pi in Buffer- |                                           |                        |                             |
| G-100 Fraction          | 1.9                    | 65                                                  | 34.2                                      | 17                     | 171                         |
| Mono Q fraction         | 0.3                    | 62                                                  | 206                                       | 17                     | 1033                        |

Typically, G-100 fraction had a volume of 210 ml containing 2 mg protein with specific activity of 34 nmol Pi released/min/mg at 45°C.

For obtaining the molecular size of PP1-Arch, DE-52 fraction II, 10 ml, was concentrated by centrifugal ultrafiltration in a Centriprep 10 to a volume of less than 1 ml. The concentrated DE-52 fraction II was applied to a column of Sephadex G-100 (1.5 x 100 cm) and eluted with the same buffer as the above gel filtration column. The apparent molecular weight as compared to protein standards was 28 kDa (Figure 4, Panel B).

### Mono Q FPLC

The following procedure was performed at room temperature. A Mono Q FPLC column (0.5 x 7 cm) was equilibrated with Mono Q buffer (10 mM MES, pH 6.5, containing 35 µg/ml PMSF, 0.5 mM EDTA and 0.5 µg/ml leupeptin). G-100 fraction was centrifuged for 10 min at 12000 x g and the supernatant was loaded onto the Mono Q column using an FPLC superloop (Pharmacia) at a flow rate of 1 ml/min. The column was washed with 5 ml Mono Q buffer, followed by a 2 ml linear salt gradient of 0 - 170 mM NaCl in Mono Q buffer. The protein phosphatase then was eluted with a linear salt gradient consisting of 17.5 ml each of Mono Q buffer containing either 170 mM NaCl or 270 mM NaCl. Fractions, 1 ml each, were collected and assayed for protein phosphatase activity (Figure 5). Active fractions were pooled as Mono Q fraction and stored at - 20°C. Typically, Mono Q fraction, 10 ml containing 0.3 mg protein, had a specific activity of 190 nmol Pi released/min/mg at 45°C.

The summarization of purification was shown in Table 3. Overall, about 1000 fold purification of PP1-Arch from soluble fraction of *Sulfolobus solfataricus* was achieved with a 15-20% recovery of activity.

### **Purity of Mono Q fraction and identification of PP1-Arch protein**

Mono Q fraction showed multiple polypeptide bands on SDS-PAGE (Figure 6). In order to obtain peptide sequences from PP1-Arch to facilitate the cloning of its gene, it is necessary to identify the polypeptide corresponding to PP1-Arch.

#### **Renaturation following SDS-PAGE**

To prevent permanent denaturation of PP1-Arch by SDS, Mono Q FPLC fraction was mixed with 1 x SDS sample buffer and heated for 5 min at 65°C instead of the usual 100°C before it was applied to a 15% SDS polyacrylamide gel. Following electrophoresis, SDS and other contaminants that might interfere the phosphatase assay were removed by soaking. The gel was then divided into gel slices which were assayed for phosphocasein phosphatase activity. As can be seen in Figure 6, a peak of phosphocasein phosphatase activity was observed whose position corresponded to that of a 33 kDa polypeptide, which was the major band on SDS-PAGE. The activity was also divalent metal ion-stimulated, further indicating that the 33 kDa polypeptide must be PP1-Arch.

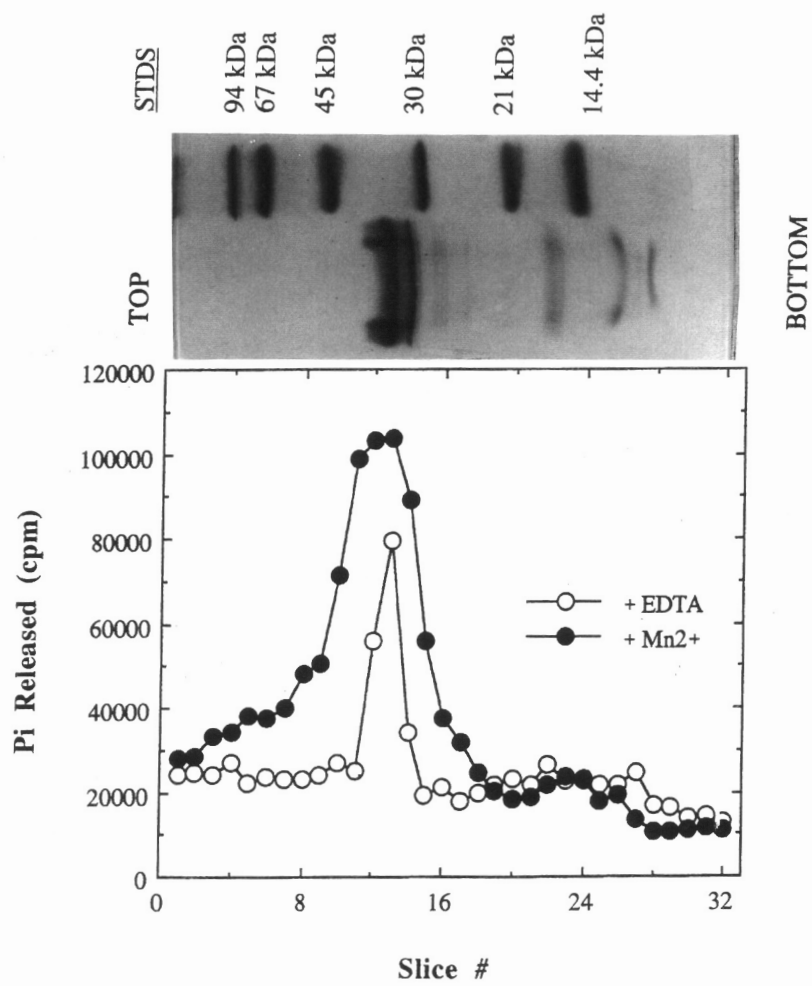
### Other experiments that identifying the major polypeptide as PP1-Arch

1. Native-PAGE: Mono Q fraction was applied to a 15% Native-polyacrylamide gel (Bollag and Edelstein, 1991). The gel was stained neutrally with 0.1%(w/v) Commassie Brilliant Blue in 25%(v/v) isopropanol and detained with 10%(v/v) isopropanol. The sections (0.2 cm x 0.5 cm) containing proteins were excised individually and homogenized with a plastic pestle in 30  $\mu$ l of phosphatase assay buffer. After soaking overnight at 4<sup>0</sup>C, these gel slice mixtures were assayed for protein phosphatase activity by adding 10  $\mu$ l of <sup>32</sup>P-phosphocasein and working up via TCA precipitation as described previously. Assay results indicated that the major protein band on the native polyacrylamide gel had protein phosphatase activity and other visible protein bands did not.

2. Isoelectric focusing: Mono Q fraction was applied to an isoelectric focusing gel. The position of the major protein band corresponded with the peak of the phosphocasein phosphatase activity (Figure 7). The estimated pI of PP1-Arch from the isoelectric focusing gel was about 5.3.

3. Mono Q FPLC: G-100 fraction, 20 ml, was applied to a Mono Q column that was equilibrated in Mono Q buffer. The column was washed with 5 ml of the same buffer and eluted with a linear salt gradient consisting of 15 ml each of Mono Q buffer containing either 150 mM NaCl or 300 mM NaCl. Fractions, 1 ml each, were collected and assayed for protein phosphatase activity. Portions, 20  $\mu$ l, of each fraction were applied to a 15% SDS-polyacrylamide gel. As can be seen in Figure 8, the phosphocasein phosphatase activity coeluted with the 33 kDa polypeptide seen on SDS-

**Figure 6. Renaturation of PP1-Arch on SDS-PAGE.** Mono Q fraction, 15  $\mu$ l, containing 7.5  $\mu$ g protein, and 4 x SDS sample buffer, 6  $\mu$ l, were mixed and heated 5 min at 65°C. The mixture was subjected to a 20% SDS-polyacrylamide gel. After electrophoresis, the gel was soaked against 50 mM MES pH 6.5, containing 5 mM EDTA for 30 minutes, followed by 50 mM MES, pH 6.5, for 30 minutes. The gel was then divided into 32 slices, and each slice was homogenized, soaked in phosphatase assay buffer, and assayed for phosphocasein phosphatase activity as described under "Specific Procedure". Shown are the commassie blue stained proteins in Mono Q fraction from a second sample run in the same gel and a plot of the phosphocasein phosphatase activity as a function of gel slice number.



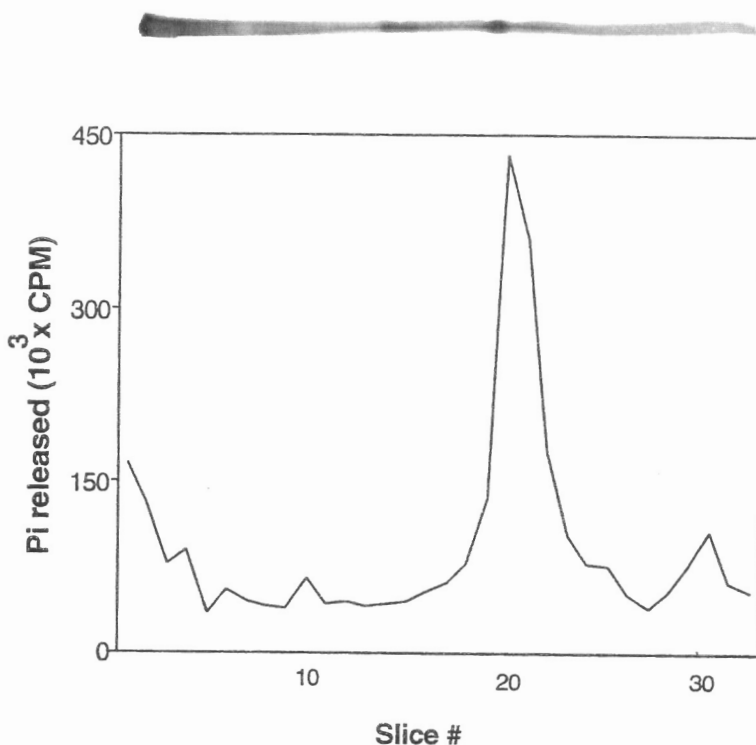
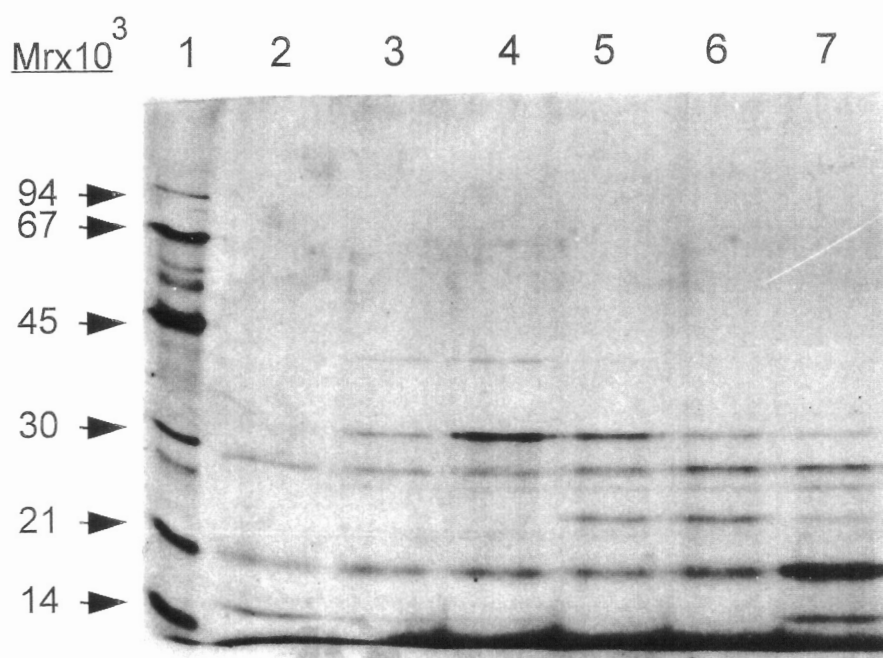
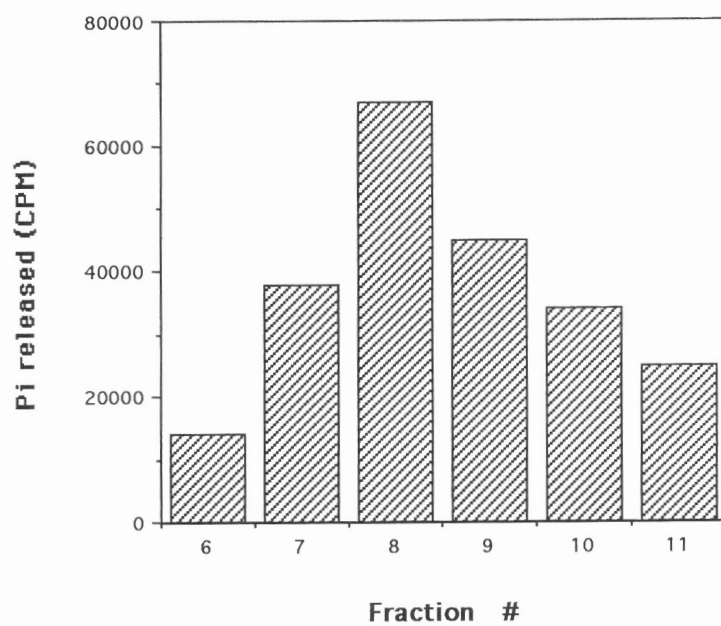


**Figure 7. Correspondence of the major polypeptide in Mono Q fraction with the peak of protein phosphatase activity on an isoelectric focusing gel.** Mono Q fraction, 20  $\mu$ l containing 2  $\mu$ g protein, and 6 x sample buffer (50%(v/v) glycerol, 6%(v/v) Bio-Rad Ampholytes, pH 4-6), 6  $\mu$ l, was mixed and loaded to an isoelectric focusing tube gel (0.15 x 7.2 cm). Isoelectric focusing was performed as described under "General procedures". The gel was divided into 0.15 cm x 0.2 cm slices using a Bio-Rad Gel Slicer (Model 192). The slices were placed in Eppendorf tubes and soaked for 4 hours at 4°C in 30  $\mu$ l of phosphatase assay buffer. Next, 6  $\mu$ l of  $^{32}$ P-labelled phosphocasein was added to the gel slice mixture and incubated for 2 hours at 45°C. After the incubation, 140  $\mu$ l of 20%(w/v) TCA was added to the assay mixture. The reaction mixture was centrifuged for 3 min at 12000 x g and 70  $\mu$ l of TCA-soluble supernatant was counted for radioactivity. Shown is a plot of the phosphocasein phosphatase activity of each gel slice along with the staining pattern of a second gel running parallel.

**Figure 8. Coelution of PP1-Arch activity and the 33 kDa polypeptide on a Mono Q column.** G-100 fraction, 20 ml, was applied to a Mono Q column which was equilibrated in Mono Q buffer. The column was washed the same buffer and eluted with a linear salt gradient consisting of 15 ml each of Mono Q buffer containing either 150 mM NaCl or 300 mM NaCl. Fractions, 1 ml, were collected and assayed for protein phosphatase activity. Portions, 20  $\mu$ l each, of fraction 6 through fraction 10 were applied to a 15% SDS-polyacrylamide gel. Lane 1: molecular weight standard, Lane 2: Fraction #6, Lane 3: Fraction #7, Lane 4: Fraction #8, Lane 5: Fraction #9, Lane 6: Fraction #10, Lane 7: Fraction #11.



PAGE. This suggested that the 33 kDa polypeptide was PP1-Arch.

## **Characterization of PP1-Arch**

### Physical Properties

#### **1. Cellular location**

PP1-Arch activity was found in soluble fraction of *Sulfolobus solfataricus*. No activity was detected in either the pellet fraction or growth media of *Sulfolobus solfataricus*. Since the optimal pH for the phosphocasein phosphatase activity was 6.5, and the fact that *Sulfolobus solfataricus* maintains a neutral cytoplasmic pH, this implicates the cellular location of PP1-Arch as the cytosol.

#### **2. Molecular size**

The apparent molecular size of PP1-Arch was determined as 28 kDa on gel filtration chromatography (Figure 4, Panel B). The subunit molecular weight was 33 kDa on SDS-PAGE (Figure 6). This indicates that PP1-Arch is a monomer.

#### **3. Heat stability**

As shown in Figure 9, PP1-Arch retained nearly full ( $\geq 70\%$ ) activity when heated a period of 30 min at temperatures ranging from 50°C to 95°C.

#### **4. Peptide sequences**

##### **(1) N-terminal sequence of PP1-Arch**

Mono Q fraction was applied to a SDS-polyacrylamide gel and the proteins thus resolved were transferred to a PVDF membrane. The portion

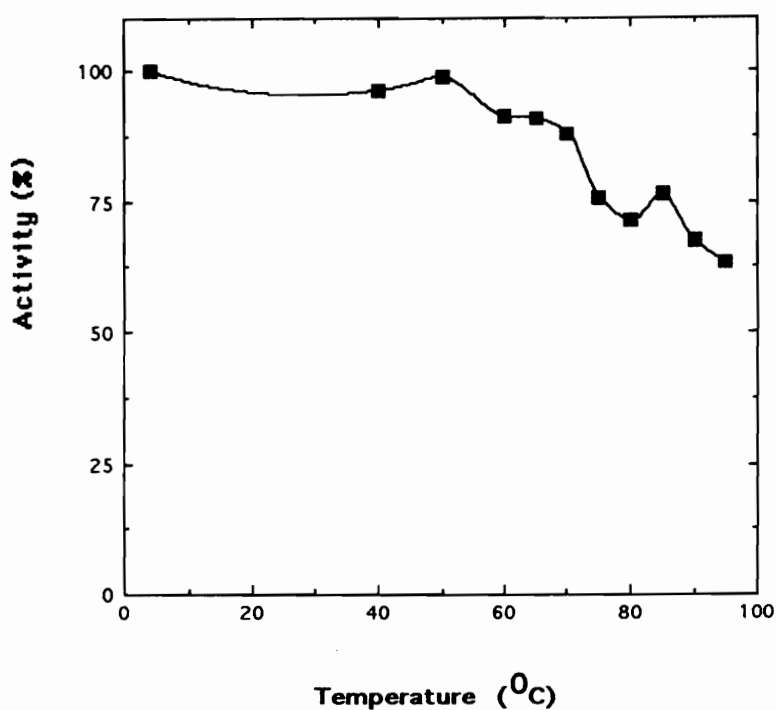


**Figure 9. Heat stability of PP1-Arch.** Portions, 0.5 ml each containing 0.36 mg protein, of DE-52 fraction II, were incubated for 30 min at the temperatures ranging from 40°C to 95°C as indicated. After incubation, the sample was clarified by centrifugation (10 min, 12000 x g), and the supernatant was assayed for phosphocasein phosphatase activity under standard conditions. Shown is a plot of activity of the supernatant as a function of temperature.

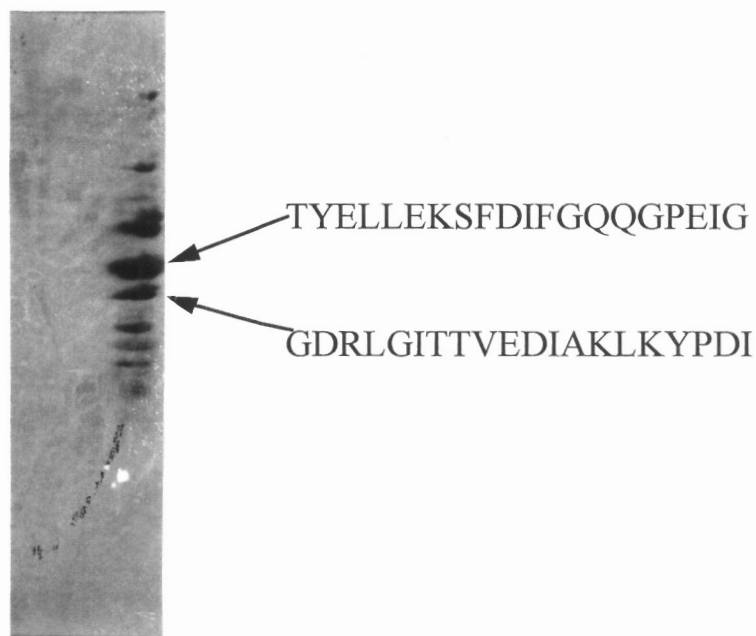


**Figure 10. Partial digestion of PP1-Arch with V8 proteinase.** Mono Q fraction, 20  $\mu$ l containing 10  $\mu$ g protein, was applied to a 15% SDS-polyacrylamide gel and electrophoresed under standard conditions. The gel was stained with Commassie blue and the section containing PP1-Arch was excised. The gel slice was subjected to another 20% SDS-polyacrylamide gel and PP1-Arch was partially digested with V8 proteinase as described in "Specific procedures". The resulting peptides were transferred to a PVDF membrane and the membrane was then stained with Commassie blue as described previously. Sections of the membrane containing two of the most prominent peptides were excised and subjected to amino acid sequence analysis. Shown are Commassie blue stained peptides on the PVDF membrane.

of the membrane containing PP1-Arch was excised and subjected to amino acid sequence analysis. The N-terminal sequence of PP1-Arch was determined to be NH<sub>2</sub>-MNIETYELLEK.

## (2) Internal peptide sequences of PP1-Arch

Mono Q fraction, 20  $\mu$ l, was applied to a 15% SDS-polyacrylamide gel and electrophoresed under standard conditions. The gel was stained with Commassie Brilliant Blue and the section containing PP1-Arch was excised.

The gel slice was partially digested by V8 proteinase as described in methods. The resulting peptides were resolved by SDS-PAGE and electroblotted onto a PVDF membrane. After being stained with Commassie Brilliant Blue, several peptides were apparent. Two of the major peptide bands were excised from the membrane and subjected to amino acid sequence analysis. Their amino acid sequences are shown in Figure 10. The last eight residues of the N-terminal sequence and the first eight residues of the top peptide on Figure 10 match exactly, indicating that they can be combined to give the first twenty-five amino acids of PP1-Arch: NH<sub>2</sub>-MNIETYELLEKSFDIFRQQGPFIG.

## Catalytic properties

### 1. Stimulation of PP1-Arch activity by divalent metal ions

Previously, it was observed that PP1-Arch activity was dramatically stimulated by the presence of divalent metal ions, such as Mn<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup>, and Mg<sup>2+</sup>. Using DE-52 fraction, the K<sub>act</sub> for Mn<sup>2+</sup> and Ni<sup>2+</sup> were estimated to be 0.05 mM and 0.1 mM, respectively (Figure 11).

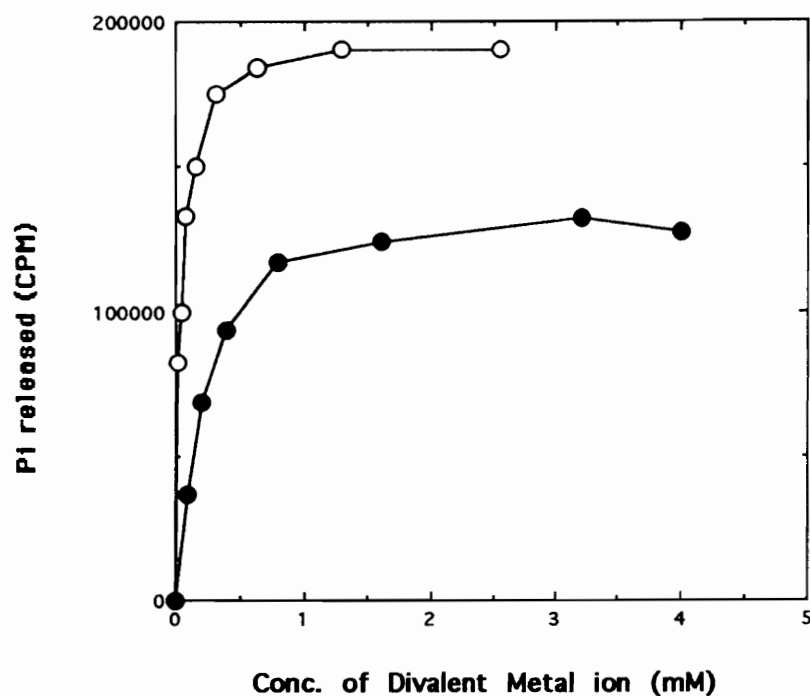


**Figure 11. Activation of PP1-Arch by  $\text{Mn}^{2+}$  and  $\text{Ni}^{2+}$ .** DE-52 fraction, 5  $\mu\text{l}$  containing 6.5  $\mu\text{g}$  protein, was assayed under standard conditions with the exception that the identity and concentration of the divalent metal ion present was varied as indicated. Shown is the phosphocasein phosphatase activity as a function of the concentration of  $\text{MnCl}_2$  (○) and  $\text{NiCl}_2$  (●). All values were corrected using controls in which 5 mM EDTA was substituted for the divalent metal ions.

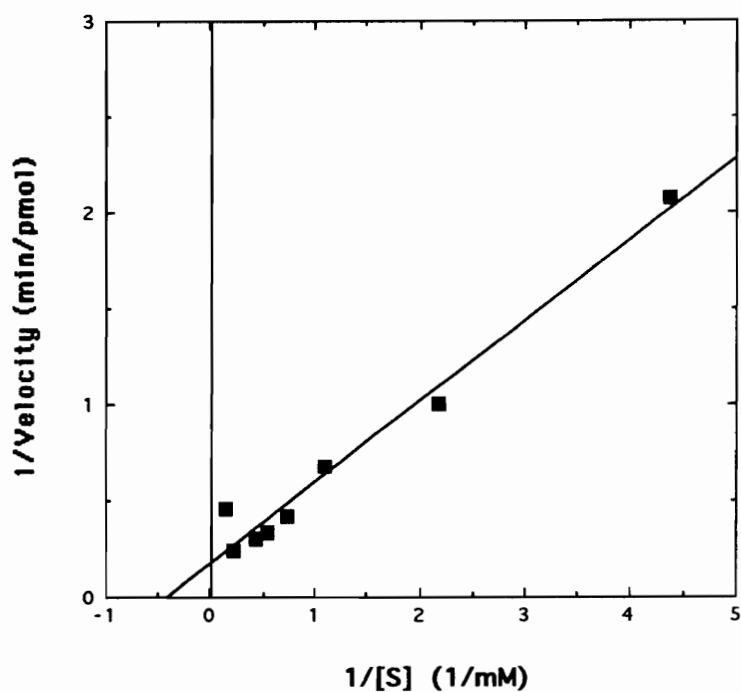


**Figure 12.  $V_{\max}$  and  $K_m$  of dephosphorylation of phosphocasein.** A portion, 5 ng, of Mono Q fraction was assayed under standard conditions with the exception that the concentration of phosphocasein was varied as indicated. Shown is double reciprocal plot of 1/velocity (1/V) and 1/substrate concentration (1/S).

## 2. $K_m$ and $V_{max}$ for dephosphorylation of phosphocasein

Using phosphocasein as the substrate,  $K_m$  and  $V_{max}$  of PP1-Arch were determined as shown in Figure 12 . The  $K_m$  was about 2.4  $\mu$ M phosphate bound to casein and  $V_{max}$  was 2.2  $\mu$ mol Pi released/min/mg at 25°C.

## 3. Effects of okadaic acid and sodium molybdate on PP1-Arch activity

Previously, it was observed that PP1-Arch activity was not affected significantly by a wide range of compounds known to serve as second messengers in eukaryotic organisms, or inhibitors or activators of some eukaryotic protein phosphatases (Kennelly *et al.*, 1993), including cyclic nucleotides,  $Ca^{2+}$ /CaM, polyamines, polyanions, the acid phosphatase inhibitor tartate, the alkaline phosphatase inhibitor tetramisole, and the tyrosine phosphatase inhibitor vanadate. NaF, EDTA, phosphate and pyrophosphate did inhibit PP1-Arch activity. Okadaic acid, a potent PP1 and PP2A inhibitor (Takai, 1988), sodium molybdate, a tyrosine phosphatase inhibitor, had no significant effects on PP1-Arch activity. In addition, in Dr. Kennelly's laboratory, A. J. Cameron demonstrated that DEPC inhibited PP1-Arch activity (unpublished results).

## 4. Substrate specificity

$^{32}$ P-labeled casein was initially used to demonstrate the presence of PP1-Arch activity in the soluble fraction of *Sulfolobus solfataricus*. Kennelly et al. (1993) had shown that, in addition to casein, glycogen phosphorylase, phosphorylase kinase, and histones all were exogenous substrates for PP1-Arch. Myosin light chain had been shown to be a substrate for both type 1 and type 2 protein phosphatases *in vitro* (Cohen, 1989). I therefore

assayed DE-52 fraction with  $^{32}\text{P}$ -labeled myosin light chain under standard assay conditions, the results of which demonstrated that myosin light chain was indeed an exogenous substrate for PP1-Arch, and the dephosphorylation by PP1-Arch at comparable level as casein.

## **Cloning and sequencing PP1-Arch gene**

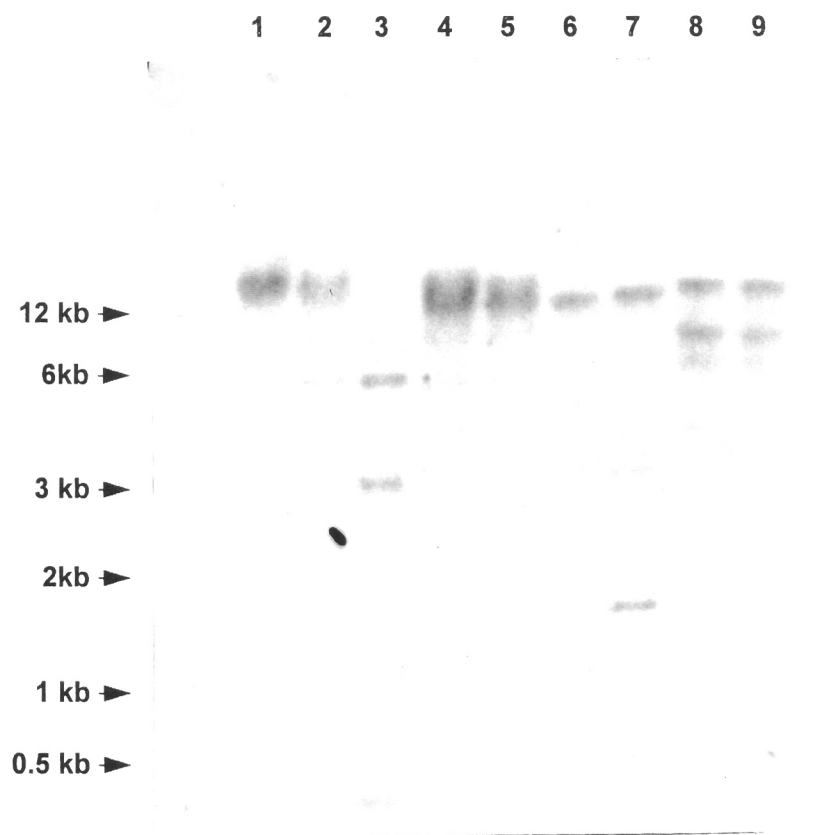
### Southern Blotting

To assist in choosing an appropriate restriction enzyme for constructing the genomic DNA library of *Sulfolobus solfataricus*, four different restriction enzymes were used to digest *Sulfolobus solfataricus* genomic DNA. Figure 13 shows the results of a Southern blot analysis in which  $^{32}\text{P}$ -labeled oligonucleotide 1 (Table 1) was used as probe. The results indicated that *EcoRI* generated DNA fragments within the desired size range ( $\leq 10$  kb) that hybridized with this probe.

### Construction of genomic DNA libraries of *Sulfolobus solfataricus*

*Sulfolobus solfataricus* genomic DNA that had been digested with either *EcoRI* or *HindIII* was ligated into pGEM-3Z that had been cleaved with the same enzyme and dephosphorylated by CIAP, then transformed into competent DH5aF' cells. In both cases the percentages of total colonies that were recombinant colonies were over 90%. To determine the average insert size for each DNA library, plasmid DNAs isolated from a dozen randomly chosen recombinant colonies were digested with the appropriate restriction

**Figure 13. Southern Blotting.** Southern blotting was performed as described under "General procedures". Briefly, genomic DNA of *Sulfolobus solfataricus*, 50  $\mu$ g or 100  $\mu$ g, was digested with 50 units of *Eco*RI, *Bam*HI, *Xba*I or *Hind*III for overnight at 37°C. Aliquots, each containing 20  $\mu$ g DNA, were loaded and analyzed by electrophoresis on a 1.0%(w/v) agarose gel. DNA fragments in the gel were denatured and transferred onto a nylon membrane. The membrane was hybridized with <sup>32</sup>P-labeled oligonucleotide 1. Shown is an autoradiogram of the hybridized membrane. All lanes contain 20  $\mu$ g genomic DNA. Lane 1: the one from undigested genomic DNA, Lane 2: the one from 100  $\mu$ g genomic DNA digested by *Eco*RI, Lane 3: the one from 50  $\mu$ g genomic DNA digested by *Eco*RI, Lane 4: the one from 100  $\mu$ g genomic DNA digested by *Bam*HI, Lane 5: the one from 50  $\mu$ g genomic DNA digested by *Bam*HI, Lane 6: the one from 100  $\mu$ g genomic DNA digested by *Xba*I, Lane 7: the one from 50  $\mu$ g genomic DNA digested by *Xba*I, Lane 8: the one from 100  $\mu$ g genomic DNA digested by *Hind*III, Lane 9: the one from 50  $\mu$ g genomic DNA digested by *Hind*III.



enzyme and the sizes of their insert were determined by agarose electrophoresis. This analysis showed that the average insert size was about 2.5 kb for the library in which *EcoRI* was chosen as the restriction enzyme, and 4.1 kb for the one in which *HindIII* was chosen as the restriction enzyme. The average insert sizes thus determined were used to calculate the number of recombinant colonies that needed to be screened in order to cover the entire genome of *Sulfolobus solfataricus*.

### Polymerase chain reaction

Using the partial amino acid sequence information obtained earlier, we decided to take advantage of the fact that our possession of both N-terminal and an internal peptide sequence would allow us to know the orientations of these two segments of protein sequence information one to another. This facilitated using PCR to amplify the DNA fragment that encoded the portion of the gene coding the region linking those two segments. We designed three degenerate oligonucleotide primers (Table 1, Oligonucleotides 2, 3, and 4) in which two nested primers, oligonucleotide 2 and 3, were derived from the N-terminal amino acid sequence with a direction of 5' to 3' of sense strand and oligonucleotide 4 was derived from the internal peptide with a direction of 5' to 3' of antisense strand. We selected a "touchdown" method for PCR in which the annealing temperature is gradually decreased in 1<sup>o</sup>C increments to "titrate" binding of degenerate primers to the highest affinity sites. As indicated in Figure 14, "touchdown" PCR produced a 560 bp PCR product when oligonucleotides 2 and 4 were used as primers. If this PCR

product was derived from the gene for PP1-Arch, we reasoned that PCR amplification with oligonucleotide 3 and 4 as primers should yield a new PCR product 39 bp shorter than the first one. As shown in Figure 14, when the first round PCR product was amplified with these primers (oligonucleotides 3 and 4), a single PCR product of 520 bp was produced, which as predicted was 40 bp shorter than the first PCR product.

The first round PCR product was cloned into pGEM-T vector (Promega) to generate a plasmid called pJL-1. pJL-1 was sequenced using vector primer T7, SP6, and degenerate gene primer (oligonucleotide 1). In each case, the results showed that the amino acid sequence predicted by the PCR product matched that of the NH<sub>2</sub>-terminal region of PP1-Arch.

#### Screening of *Sulfolobus solfataricus* genomic DNA library

In order to obtain a larger and, hopefully, complete clone of PP1-Arch, the *Eco*RI library of *Sulfolobus solfataricus* genomic DNA was probed with both <sup>32</sup>P-labeled oligonucleotide 1 and the "touchdown" PCR product (Figure 15). When oligonucleotide 1 was used as probe, 25 "positive" colonies were selected. Further analysis revealed that three of them encoded part of PP1-Arch. When the "touchdown" PCR product was used as probe, 4 positive colonies were selected. DNA sequencing revealed that all four encoded part of PP1-Arch and three of those four colonies were the same as the ones identified using oligonucleotide 1 as probe. DNA sequencing data indicated that none of these positive recombinants contained the complete gene for PP1-Arch, because there was an *Eco*RI site at amino acid 223 of PP1-Arch.

**Figure 14. 'Touchdown' PCR.** 'Touchdown PCR' was performed using 1 µg *Sulfolobus solfataricus* genomic DNA as template and 250 pmol of oligonucleotide 2 and oligonucleotide 4 as primers according to the description under "General procedure". A second PCR was performed under standard conditions using 1 µl of the 'touchdown' PCR reaction mixture as template and 100 pmol of oligonucleotide 3 and oligonucleotide 4 as primers.

## N-terminal peptide sequence

M N I E E T Y E L L E K S F D I F G  
ATG AAY ATH GAR GAR ACN TAY GAR YTN YTN GAR AAR WSN TTY GAY ATH TTY GGN

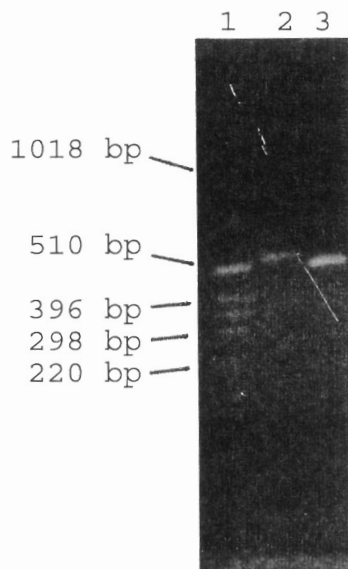
primer 1  
Q Q G P F I G  
CAR CAR GGN ATH TTY ATH GGN

primer 2

## Internal peptides sequence

G D R L G I T T V E D I A K L K Y P  
GGN GAY MGN YTN GGN ATH ACN ACN GTN GAR GAY ATH GCN AAR YTN AAR TAY CCN  
D I CGN TTY RAN TTY ATR GGN  
GAY ATH  
CT

primer 3



Lane 1: 1Kb Ladder

Lane 2: Touchdown PCR product  
(primer 1 and primer 3)

Lane 3: Amplification of touchdown  
PCR product (primer 2 and  
primer 3)

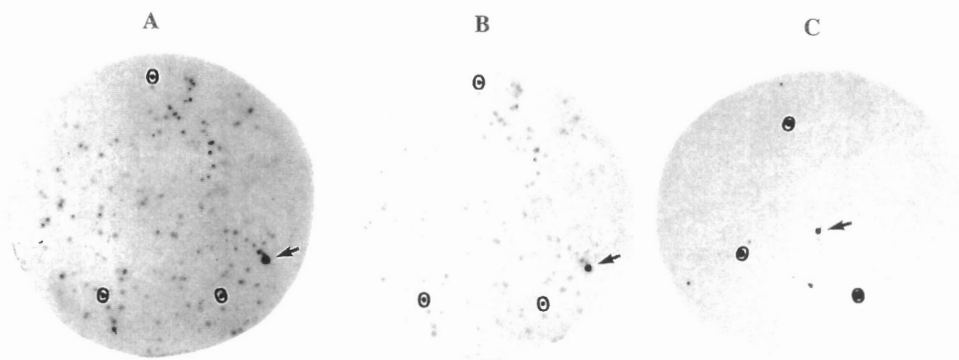


**Figure 15. Screening of *Sulfolobus solfataricus* genomic DNA library.** The preparation and hybridization of nitrocellulose membranes containing the *Sulfolobus solfataricus* genomic DNA library was performed as described under "General Procedures". Shown is autoradiogram of one of the filters containing positive signal (▲). Each filter was orientated using the three circled dots that were placed upon them using fluorescent ink (●). Panel A: Oligonucleotide 1 as probe for the *Sulfolobus solfataricus* genomic DNA library in which *Eco*RI was chosen as the restriction enzyme, Panel B: 'Touch down' PCR product as probe for the *Sulfolobus solfataricus* genomic DNA library in which *Eco*RI was chosen as the restriction enzyme (reprobing of filter used in Panel A), and Panel C: Probe is PCR product in which genomic DNA served as template and oligonucleotide 6 and oligonucleotide 13 served as primers for the *Sulfolobus solfataricus* genomic DNA library in which *Hind*III was chosen as the restriction enzyme.

To get a complete clone of PP1-Arch, a second library of *Sulfolobus solfataricus* genomic DNA was constructed using *Hind*III as the restriction enzyme. Oligonucleotide 6 and oligonucleotide 13 were used as PCR primers to amplify the region between amino acid 65 and amino acid 209 of PP1-Arch from *Sulfolobus solfataricus* genomic DNA, and this PCR product was used as a probe to screen the *Hind*III library of *Sulfolobus solfataricus* genomic DNA. Two colonies were selected and DNA sequencing indicated that one contained the intact gene for PP1-Arch. This recombinant plasmid was called pJL-2.

#### DNA Sequence of PP1-Arch gene:

PP1-Arch gene was completely sequenced on both strands (Figure 16). The results are summarized in Figure 17. At position -25 of the sequence, there is a potential promoter which matches with the consensus promoter sequence (TTTA/TAA) for known *Sulfolobus* genes (Brown *et al.*, 1989)

The analysis of base composition, amino acid composition, molecular size, and codon usage of PP1-Arch was performed using PCGENE, and the results are shown in Table 4 and Table 5. The analysis of the predicted properties of the PP1-Arch protein was obtained using DNASTAR, the results of which are shown in Figure 18.

Archaeobacteria are known to be divergent from eukaryotes, but evolutionarily closer than eubacteria to eukaryotes (Woese, 1994). In order to determine whether there is a sequence homology exists between PP1-Arch and protein phosphatases from eukaryotic organisms, a comparison was made

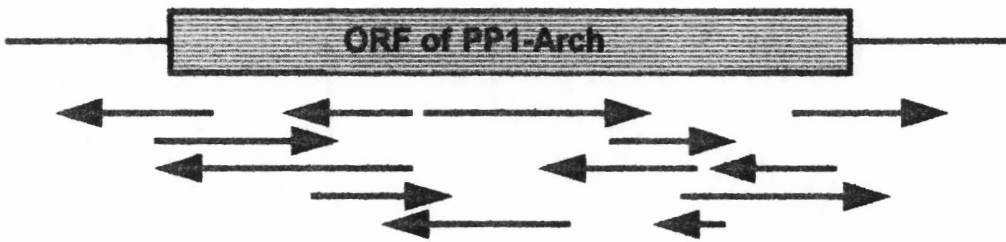


**Figure 16. DNA sequencing strategy for PP1-Arch.** The figure summarizes the strategy used to sequence the PP1-Arch gene on both strands. Each arrow represents the region covered by a particular sequencing experiment, and the area sequenced: Left facing arrows: antisense strand; Right facing arrow: sense strand.

**Figure 17. DNA-derived amino acid sequence of PP1-Arch.** Shown is the nucleotide and DNA-derived amino acid sequence of PP1-Arch. Underlined areas indicate the positions of peptide sequences that were obtained earlier.

5' - GCACTGCTTCTCCAAATCCAACCATACCATTGATGTCA  
CTACCCTTACCAATATCATAACCGATGCCCATGTTGCACTGCCTTTTTTCAGTTTGTGCTA  
GGACTATAGGTTCTATTTGGGAGATTTTTGTGCATACAATATCTTAAACAAATAACTCTAA  
AATACCTTCATCAAATAGGGCTATATAATTTTTTAAATAGAACACAATATACCACAATATT  
GTG AAC ATT GAA GAA ACG TAT GAG TTG TTA GAA AAA TCA AAG GAT  
M N I E E T Y E L L E K S K D  
ATA TTT AGA CAG CAA GGA CCA TTT ATT GGA GAA TAT AAA GAA GAC  
I F R Q Q G P F I G E Y K E D  
AAG GTG GTT TTC GTT GGA GAT ACG CAT GGT GCA ATT AAT GTA ACC  
K V V F V G D T H G A I N V T  
GAA TAT GTA TTT AGA AAA TTT TAC GAT AAT GTT GAT TTG ATT GTG  
E Y V F R K F Y D N V D L I V  
TTC CTA GGA GAT TAT GTT GAT AGG GAA CCA CAG ACA GGT GTA GAA  
F L G D Y V D R E P Q T G V E  
AAT CTA AGT TTA ATA TTA AAG AAA TTA ATA GAG AGT GAT GAA AAT  
N L S L I L K K L I E S D E N  
AAG GGC AAA ACT AAA ATA GTG GTA CTT AGA GGG AAT CAT GAA AGT  
K G K T K I V V L R G N H E S  
CCA CTT ACA AAC TTT CAT TAT GGA TTT TTT GAA GAA CTA AAG ATT  
P L T N F H Y G F F E E L K I  
AAA ACT GAA GAT GCA GAA AAT GCG TAT GAG AAG TTC CGA GAT CTG  
K T E D A E N A Y E K F R D L  
TTT TCA TAT ATG CCT TAC TCA GTC CTA GTT AAT GGA TAT CTT TGT  
F S Y M P Y S V L V N G Y L C  
ATG CAT GGA GGT TTA CCT GCA ACA TTA GAG GGC GAT AGA CTA GGG  
M H G G L P A T L E G D R L G  
ATC ACG ACA GTT GAA GAT ATA GCA AAG TTA AAG TAT CCA GAT ATA  
I T T V E D I A K L K Y P D I  
AAC CCA GAT GAT CCT GTA GGT TTA CAG ATA TTA TGG AAT GAT CCA  
N P D D P V G L Q I L W N D P  
AGA GAT GGA CTA CTA GAT ATA GAT TTC TTA CCC AGT ATT AGA GGT  
R D G L L D I D F L P S I R G  
GAA GGA ATA TAC TAT TTT GGG GAA AGA GTG ACA AAT GAA TTC CTA  
E G I Y Y F G E R V T N E F L  
TCC TAT AAT AAT TTT AAA GGA ATA ATC AGA GGA CAT GAA GCA GTT  
S Y N N F K G I I R G H E A V  
GAC GGA TTT AGA ACT AAC ATG AAC GGC AAA GTT ATC ACT GTA TTT  
D G F R T N M N G K V I T V F  
TCT AGT GTA TAT CAC GGT CAA AGA TCA GGT ATT TTA TAT CAT GAT  
S S V Y H G Q R S G I L Y H D  
TAT AAT AAT AAC AAA TTC GTC CGT ATA TAT ATA AAA ATG GTG AAC  
Y N N N K F V R I Y I K M V N  
GAT CAA CTA ATA GAA GAG ATA GTA TAG CAGACAAAAATAAAAAGACGTG  
D Q L I E E I V -  
AATAATTTTAGTGAAAATAAAAAATCCATTACTACTTATTCTCGTTCTTGCTCTCTGGTT  
TCTGTTCAACGACTAATTCCTTTT-3'

**Table 4. Base composition of the PP1-Arch gene and predicted amino acid composition and molecular weight of PP1-Arch.**

| Base composition              |               |
|-------------------------------|---------------|
| A (Adenine)                   | = 333 (37.4%) |
| C (Cytosine)                  | = 260 (29.4%) |
| G (Guanine)                   | = 186 (21%)   |
| T (Thymine)                   | = 103 (11.6%) |
| Total number of bases: 882    |               |
| Amino acid composition        |               |
| Ala 6                         | Cys 1         |
| Arg 13                        | Gln 6         |
| Asn 20                        | Glu 25        |
| Asp 22                        | Gly 25        |
| His 7                         | Ile 24        |
| Leu 26                        | Lys 19        |
| Met 5                         | Phe 18        |
| Pro 10                        | Ser 11        |
| Thr 13                        | Trp 1         |
| Tyr 18                        | Val 23        |
| Total number of residues: 293 |               |
| Molecular weight              |               |
| 33820 kDa                     |               |

**Table 5. Codon usage in the PP1-Arch gene.**

| AA   | Codon | Number | AA    | Codon | Number |
|------|-------|--------|-------|-------|--------|
| A    | GCG   | 1      | C     | UGU   | 1      |
| A    | GCA   | 5      | C     | UGC   | 0      |
| A    | GCU   | 0      | D     | GAU   | 20     |
| A    | GCC   | 0      | D     | GAA   | 2      |
| E    | GAG   | 5      | F     | UUU   | 12     |
| E    | GAA   | 20     | F     | UUC   | 6      |
| G    | GGG   | 3      | H     | CAU   | 6      |
| G    | GGA   | 12     | H     | CAC   | 1      |
| G    | GGT   | 7      | I     | AUA   | 14     |
| G    | GGC   | 3      | I     | AUU   | 7      |
| K    | AAG   | 8      | I     | AUC   | 3      |
| K    | AAA   | 11     | L     | UUG   | 2      |
| M    | AUG   | 4      | L     | UUA   | 11     |
| N    | AAU   | 13     | L     | CUG   | 1      |
| N    | AAC   | 7      | L     | CUA   | 9      |
| P    | CCG   | 0      | L     | CUU   | 3      |
| P    | CCA   | 6      | L     | CUC   | 0      |
| P    | CCU   | 3      | Q     | CAG   | 3      |
| P    | CCC   | 1      | Q     | CAA   | 3      |
| R    | AGG   | 1      | S     | UCG   | 0      |
| R    | AGA   | 10     | S     | UCA   | 4      |
| R    | CGG   | 0      | S     | UCU   | 1      |
| R    | CGA   | 1      | S     | UCC   | 1      |
| R    | CGU   | 1      | S     | AGU   | 5      |
| R    | CGC   | 0      | S     | AGC   | 0      |
| T    | ACG   | 3      | V     | GUG   | 6      |
| T    | ACA   | 5      | V     | GUA   | 8      |
| T    | ACU   | 4      | V     | GUU   | 8      |
| T    | ACC   | 1      | V     | GUC   | 2      |
| W    | UGG   | 1      | Y     | UAU   | 15     |
| Stop | UAG   | 1      | Y     | UAC   | 3      |
| Stop | UAA   | 0      | Start | AUG   | 0      |
| Stop | UGA   | 0      | Start | GUG   | 1      |

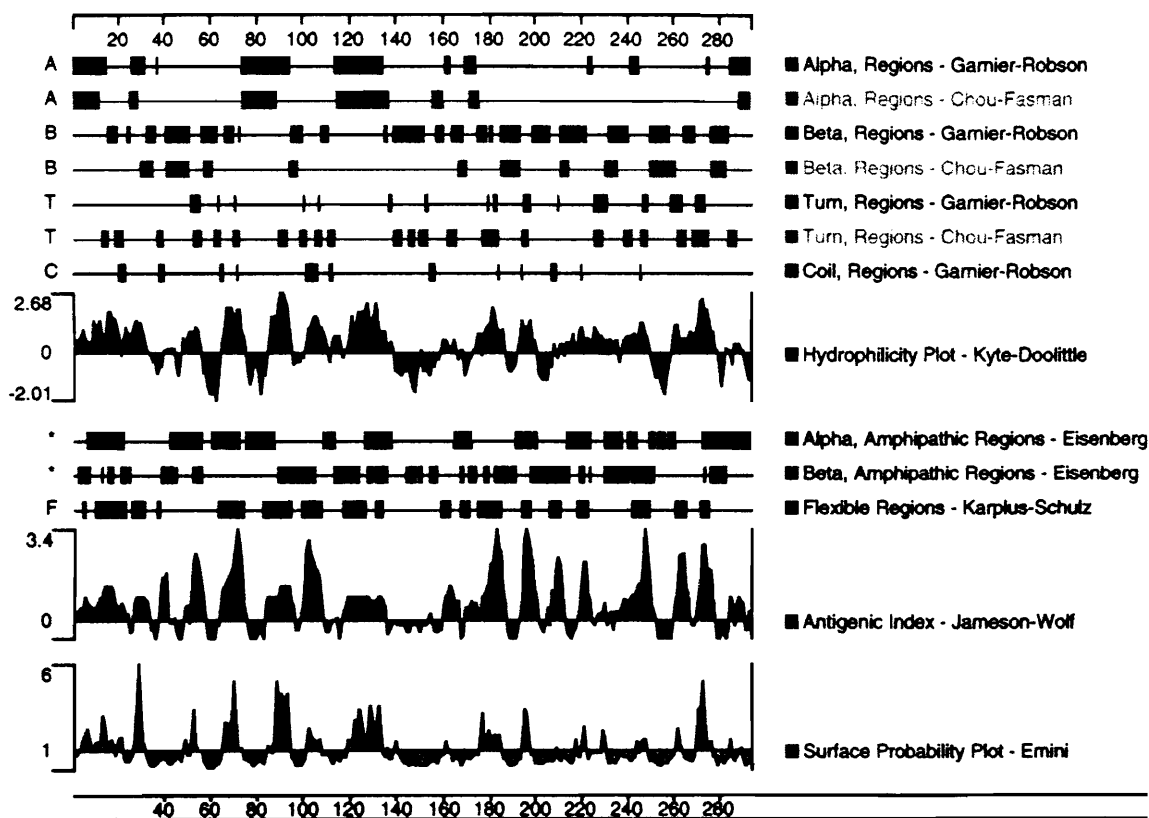


**Figure 18. Computer analysis of the predicted physical properties of PP1-Arch.**

**Figure 19. Homology between PP1 Rb, PP2A S. c., PP2B Rt, PP-λ and PP1-Arch.** Shown are the known or predicted amino acid sequences of the following: PP1 Rb: the catalytic subunit of PP1 from rabbit, PP2A S. c.: the catalytic subunit of PP2A from *Saccharomyces cerevisiae*, PP2B Rt: the catalytic subunit of PP2B from rat, PP-λ: protein phosphatase from Bacteriophage λ.

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

MNI[E]ETYE-LLEKSKS[K]DI[F]RQ[Q]GPPIGEYK[E]DK[V]VFGDTH  
QLTE[NE]IRGLCLCKMAV[DV]LQPE-ENVKPI-NAVPTICGDIH  
P[LE]E[DD]V[AR]LCKMAV[DV]LQPE-ENVKPI-NAVPTICGDIH  
RLE[ES]SVALRIITEGAS[IL]ROE-KNLLDI-DAPVTVCGDH  
MR-----YYEKIDGSKYRN-----I W V V G D L H

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

GAINVTEYV[PF]RKP[Y]DNV[DL]-IVF-LGDYVDR[EP]Q[Q]TGVENL  
GQYDDLRLP[EY]GGPP[ES]NYL[PF]-LGDYVDRGK[QS]-L[ET]I  
GQFHDLL[EL]F[K]IGGPPC[PD]TNYL[PF]-MGDYVDRGYS-[VET]V  
GQPPDLMLK[LP]EVGGSPANTRYL[PF]-LGDYVDRGYP[S]-I[EC]V  
GCTNLMNKLDTIGPDKKDLISVGD[L]VDRGAEN-[VE]-

pp1Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

SLI[L]-K[L]IESDENK[GT]K[II]VVL[RG]NHE-SPLTNPHYGGFF  
CLL[L]AYKI-KYPP[EN]-----PPLLRGNHHCASINRI-YGGFY  
SYLVAMKV-RYP[HR]-----ITILRGNHESRQITQV-YGGFY  
LYLWALKIL-YPKT-----LPLL[RG]NHECHRLT[ET]EY-FTFK  
-CLELIT-----PRAV[RG]NHE-QMM-I-DGLS

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

EE[L]KIKT[ED]AENAYEK-PRDLP[FS]Y[PM]PYSVLVN-GYLCMHG  
DECKRRY-NIK--LWKKTPTD[CF]PNC[LP]IAAI[VDE]KI[FC]CHG  
DECKLRKYGS[N]--VWKMP[DL]P[DV]TALV[DN]KI[PC]LHG  
Q[EC]KIKY-SER--VYDACM[DA]P[DC]LP[LA]AMN[Q]Q[PF]LCVHG  
ERGN-HWL-LNGGGW-P[PN]LDYDKEILAKALAHKADELPI

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

GL-PA[TL]E[GD]RLGIT[TV]EDI--AKL-KYPP[DI]N-PDDP[V]GL  
GLSP-DLQ-----SM[EQ]I--RRIMRPTDV-PDQGLLC  
GLSPM-IE-----TIDQV--RDLNR[IQ]EV--PH[EG]PMC  
GLSPF-IN-----TLDDI--RKLDRPK[EP]-PAYGPMC  
LIELVSKD-----KKYVICHADYPPDEYFGKPV[DH]Q--

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

QILWNDP-RD-----GLLDIDFL[PS]IRGEGIYFVG-ERV[ET]T  
DLLWSDPDKD-----VQGWGEND--RGVS-PTFG-AEVV  
DLLWSDPD-D--RGWGIS[P]---RGAG-PTFG-QDIS  
DILWSDPLEDPGNEKTQEHPTHT--VRC[CS]-YFYS-YPAV  
QVIWNR-ERI-----SNSQNGIVKEIKGAD-PIFGHTPAV

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

N--EPLSYNN-----PKGVIIRGHEAV-DGPR[RT]N-M  
A--KFLHKKH-D-----LDLIRAHQV[V]V[ED]DGYEPP-A  
E--QFNHTND-----LSLIA[RA]HQLVME[EG]YSWS-H  
C--DFLQHNN-----LLSILRAHEAQDAGYRMYRK  
KPLKPA[NQ]MYIDTGAVPCGNLTLLIQVQGEA

pp1-Arch  
pp1a rab.  
pp2A yst.  
pp2Ba rat  
λ PPase

NG-----KVI[TV]V[PS]SV-YH[G]Q  
KR-----QLV[TV]TLL[PS]APNYC[GE]  
QQ-----N[V]V[TV]I[PS]APNYCYR  
SQTTPPSLIT[PS]APNYLDV

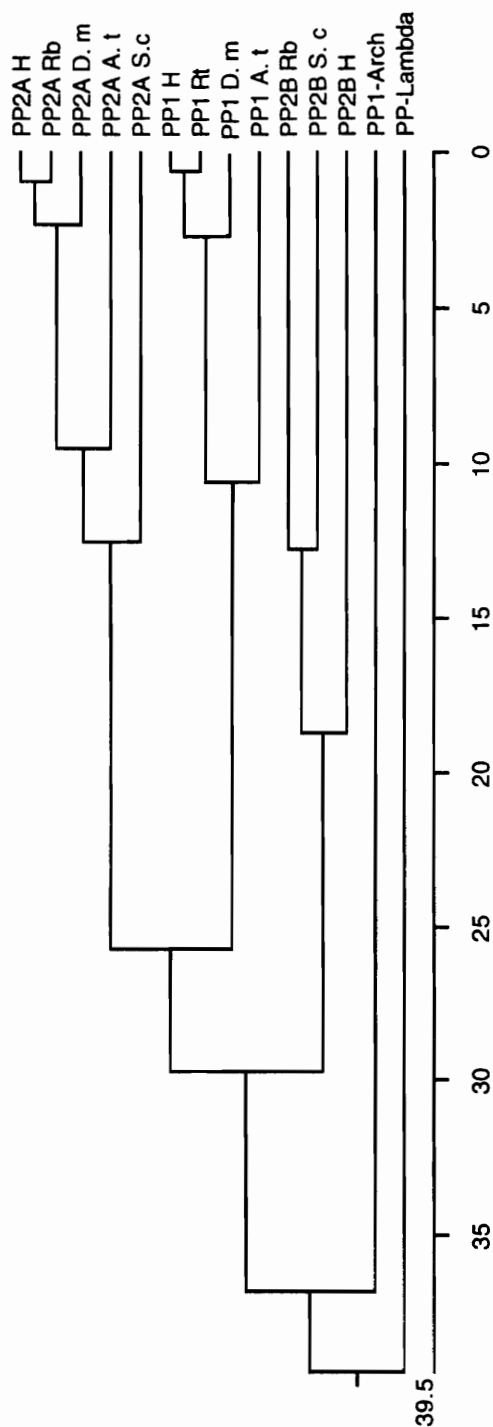


**Figure 20. Phylogenetic tree.** Shown is a dendrogram constructed using DNASTAR showing the predicted phylogenetic tree relationships among PP1-Arch; PP- $\lambda$ ; PP1 H, PP1 Rt, PP1 D.m, and PP1 A.t: the catalytic subunit of PP1 from Human, Rat, *Drosophila melanogaster*, and *Arabidopsis thaliana*, respectively; PP2A H, PP2A Rb, PP2A D.m, and PP2A A.t: the catalytic subunit of PP2A from human, rabbit, *Drosophila melanogaster*, and *Arabidopsis thaliana*, respectively; PP2B H, PP2B Rb, and PP2B S.c: the catalytic subunit of PP2B from human, rabbit, and *Saccharomyces cerevisiae*, respectively. Percent divergence is the scale.

using the following protein phosphatases: PP1 from rabbit (Berndt *et al.*, 1987), PP2A from *Saccharomyces cerevisiae* (Sneddon *et al.*, 1990), and PP2B from rat (Kuno *et al.*, 1989) as representatives for each type of protein phosphatases in the PP1/PP2A/PP2B superfamily, and PP- $\lambda$ , which is a protein phosphatase encoded by bacteriophage  $\lambda$  that is homologous to the PP1/PP2A/PP2B superfamily (Cohen and Cohen, 1989; Zhuo *et al.*, 1993). The results (Figure 19) indicate that there are three conserved motifs at the N-terminal region of the proteins within the members of PP1/PP2A/PP2B superfamily and PP- $\lambda$  that also were present in PP1-Arch. The consensus sequences for those three regions are GD(TIVL)HG, GD(YL)VDR, and RGNHE. Within the three motifs, the two histidine residues are conserved in all type 1/2A/2B protein phosphatases analyzed to date. PP1-Arch was shown to be closer to eukaryotic PP1/PP2A/PP2B family than PP- $\lambda$  as illustrated in a phylogenetic analysis (Figure 20).

### **Expression of PP1-Arch**

In order to provide a tool for future experimental studies as well as a final proof that our putative gene for PP1-Arch did encode this protein phosphatase, I decided to express PP1-Arch in *E.coli*. A recombinant plasmid (pJL-3) in which PP1-Arch gene was placed under the control of a T7 promoter was constructed as outlined in Figure 21.

### **Toxicity of the archaeobacterial phosphatase gene to *E.coli* strain BL21(DE3)**

*E.coli* strain BL21(DE3) was transformed with the recombinant

plasmid pJL-3 and grown on LB<sup>amp</sup> and M9<sup>amp</sup> plates. Compared to *E.coli* transformed with pT7-7 vector alone, there were at least 5000-fold fewer colonies visible on LB<sup>amp</sup> plates and 100-fold fewer colonies on M9<sup>amp</sup> plates plated with the *E.coli* that had been transformed with pJL-3. This indicated that expression of PP1-Arch was toxic for the growth of *E.coli*.

### Expression of the archaeobacterial phosphatase gene

A single BL21(DE3)(pJL-3) colony (see above) was grown in M9<sup>Amp</sup> medium and the expression of PP1-Arch was induced by IPTG as described previously. After induction, the cell pellet from 100  $\mu$ l of culture was lysed with 2 x SDS-sample buffer and analyzed by SDS-PAGE on a 12.5% gel. Figure 22 shows that there was an overexpressed protein that migrated at the  $R_f$  expected for PP1-Arch. Densitometry indicated that about 30% of the total proteins was this expressed protein.

### Protein phosphatase assay of the expressed enzyme

Cells containing overexpressed PP1-Arch were ruptured by sonication. The sonicated cell suspension was centrifuged and 10  $\mu$ l of the supernatant was assayed for protein phosphatase activity under standard conditions. The results, shown in Figure 22, demonstrate that high levels of phosphocasein phosphatase activity were present in cells transformed with pJL-3 and induced in IPTG, but not in controls in which cells were transformed with pT7-7. However, there was 'leaky' basal expression of PP1-Arch in uninduced cells which were transformed with pJL-3. Moreover, the

**Figure 21. Construction of pJL-3.** PP1-Arch gene was amplified by PCR in which 1  $\mu$ g of *Sulfolobus solfataricus* genomic DNA was used as template and 100 pmol each of oligonucleotide 15 and oligonucleotide 16 were used as primers. Expression vector pT7-7 and the PCR product of amplified PP1-Arch gene were digested with both *NdeI* and *SalI*. After the dephosphorylation of vector with CIAP, the vector and the digested PCR product were ligated together and transformed into competent DH5aF' cells. The resulting recombinant plasmid was named as pJL-3.

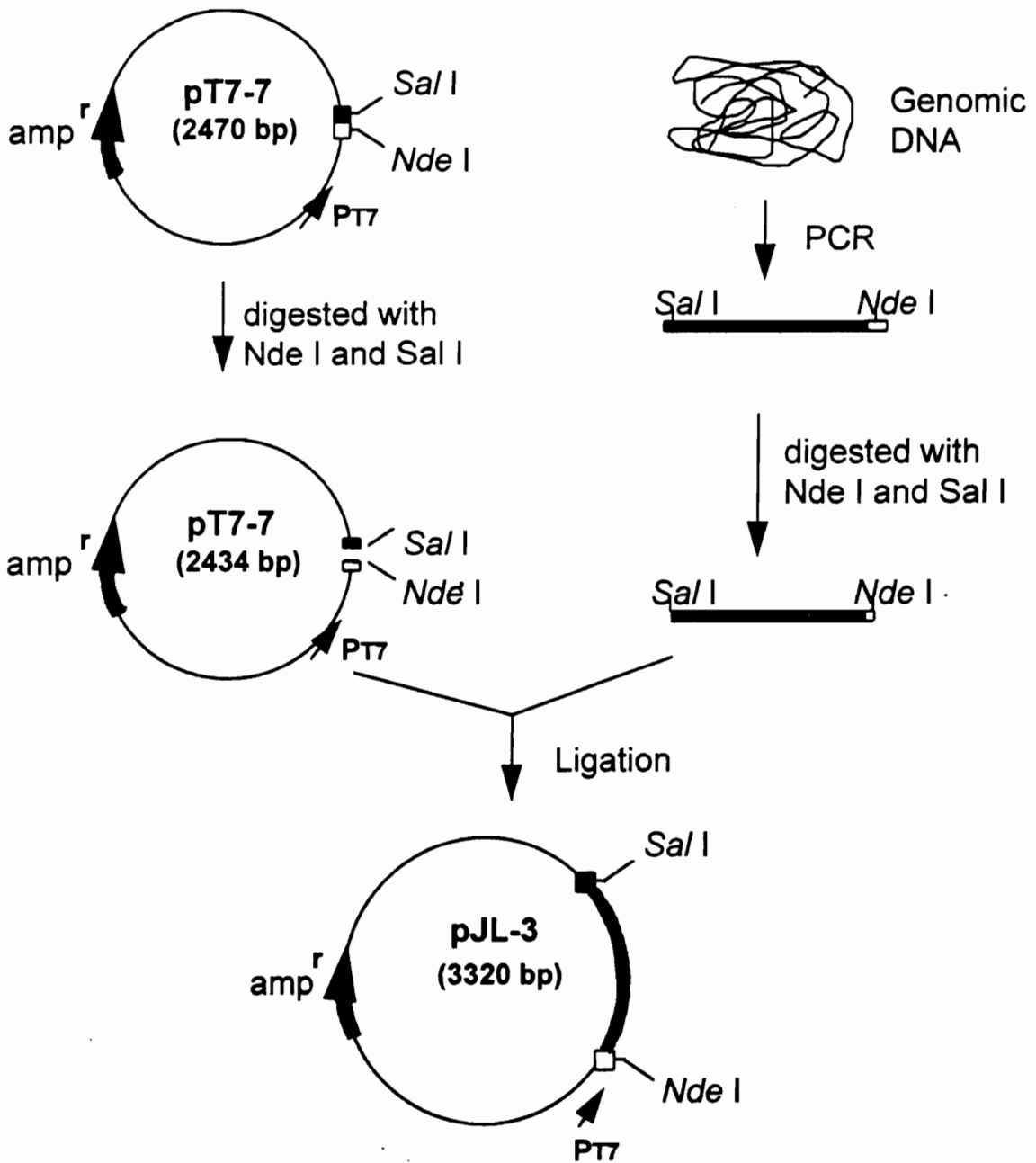


**Figure 22. Expression of PP1-Arch in BL21(DE3).** *E.coli* strain BL21(DE3) was transformed with pT7-7 or pJL-3 and expression of PP1-Arch was induced with IPTG as described under "General Procedures". The total protein in the cell pellets from 100  $\mu$ l of the culture were analyzed by electrophoresis on a 12.5% SDS-polyacrylamide gel. The remainder of the cells were ruptured by sonication. The sonicated cell suspension was centrifuged and 10  $\mu$ l of the supernatant was assayed for protein phosphatase activity under standard conditions except that, where indicated, 5 mM EDTA was substituted for  $Mn^{2+}$ . Shown are the results of protein analysis on SDS-PAGE and activity assays of soluble fraction. Lane 1: protein molecular size standard, Lane 2: pT7-7 (-IPTG), Lane 3: pT7-7 (+IPTG), Lane 4: pJL-3 (-IPTG), Lane 5 pJL-3 (+IPTG).

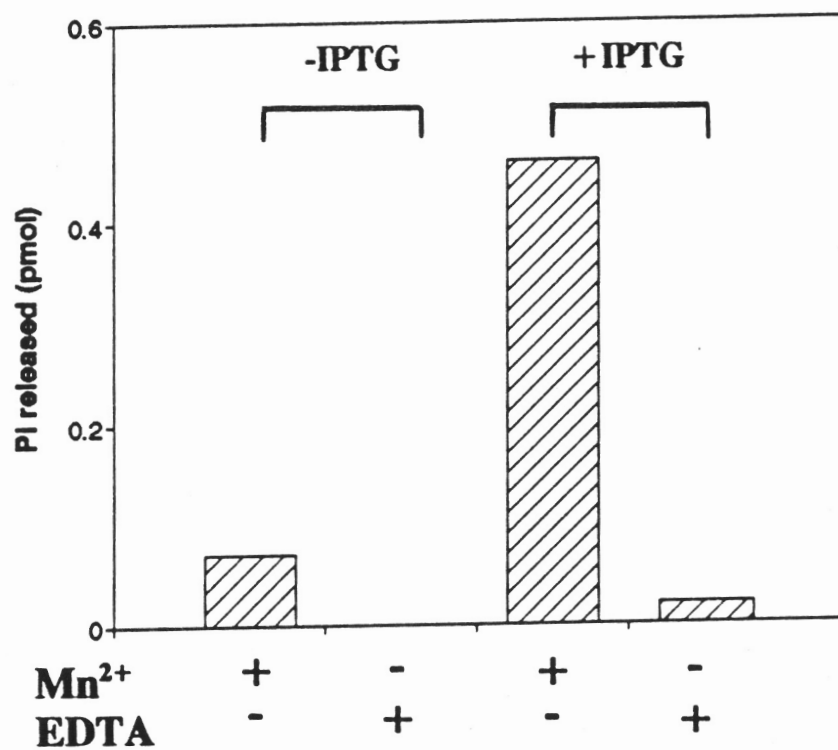
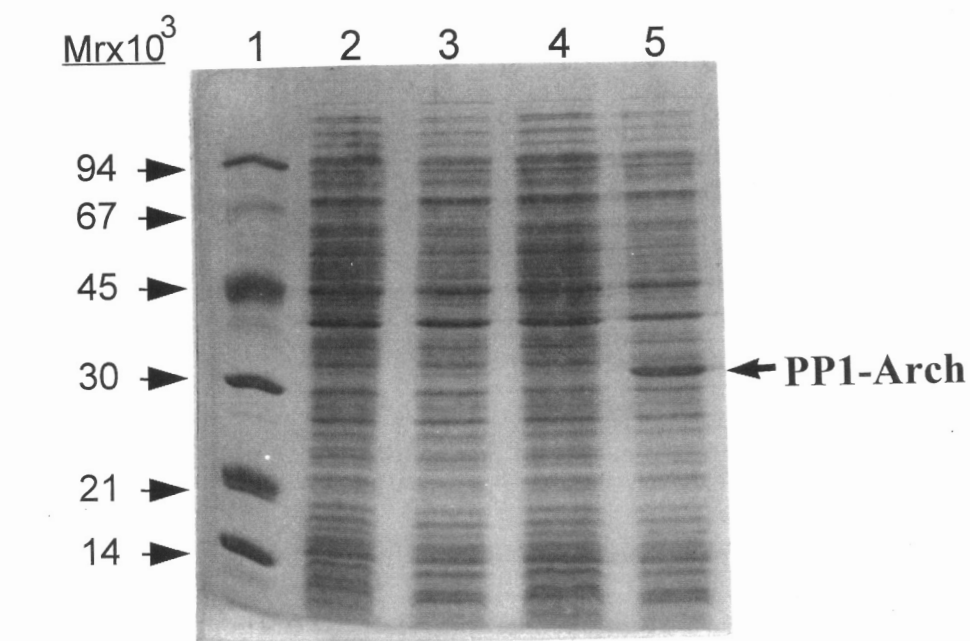
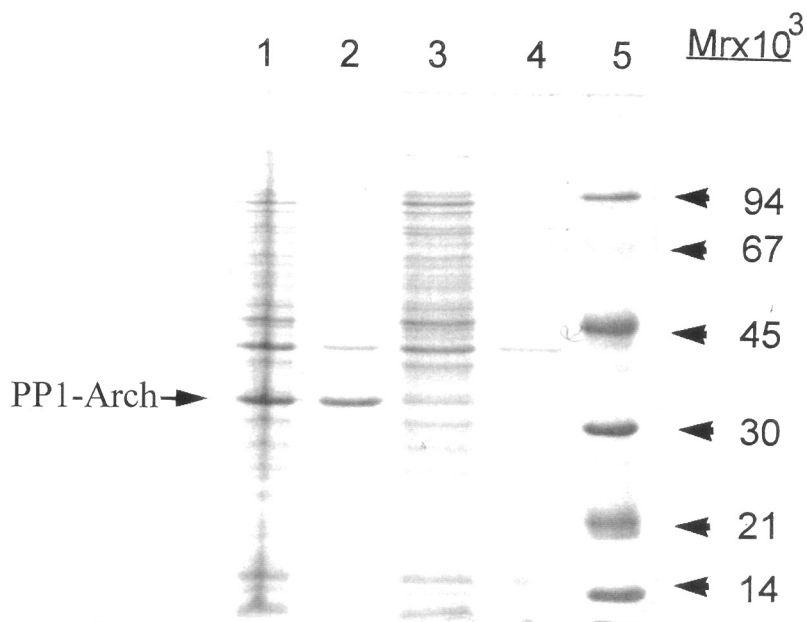


**Figure 23. Formation of Inclusion Bodies and Heat treatment.** *E.coli* strain BL21(DE3) was transformed with pJL-3 and the expression of PP1-Arch was induced with IPTG as described under "General Procedures". The cell pellets from 1 ml of culture were suspended in 4 ml of 50 mM MES, pH6.5, containing 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5  $\mu$ g/ml leupeptin and 35  $\mu$ g/ml PMSF. The cell suspension was passed through a French Press at a pressure of 25000 pounds per square inch. The homogenate was clarified by centrifugation for 5 min at 12000 x g. Following centrifugation, the pellets were suspended in 200  $\mu$ l of 1 x SDS sample buffer. A 20  $\mu$ l portion of this mixture was heated for 5 min at 100°C, then applied to a 12.5% SDS-polyacrylamide gel. A portion, 400  $\mu$ l, of the supernatant, was mixed with 2 ml of cold acetone, and the mixture was incubated for 20 min on ice. The acetone precipitated proteins were collected by centrifugation (10 min, 12000 x g) and suspended in 20  $\mu$ l of 1 x SDS sample buffer. Meanwhile, an aliquot, 400  $\mu$ l, of the homogenate supernatant was incubated for 20 minutes at 80°C. It was then centrifuged for 10 min at 12000 x g. The protein in the supernatant was precipitated with acetone and suspended in 1 x SDS sample buffer as described. Lane 1: Molecular standards, Lane 2: Proteins from cell pellet, Lane 3: Proteins from the pellet of homogenate, Lane 4: Proteins from the supernatant of the homogenate, Lane 5: Proteins from the heat-treated supernatant of the homogenate.



expressed phosphatase activity was divalent metal ion-dependent as is known to be the case for genuine PP1-Arch.

#### Formation of inclusion bodies and Heat treatment

Induced BL21(DE3)(pJL3) cells were broken with a French Press and centrifuged as described previously. The proteins in both the supernatant and the pellet were then analyzed by SDS-PAGE. The results (Figure 23) indicated that about 80% of overexpressed protein phosphatase was found in the pellet, suggesting that it was present in the form of inclusion bodies. Heat treatment (20 min at 80°C) of the supernatant caused precipitation of many *E.coli* proteins, but a small percentage of PP1-Arch remained soluble, indicating that the expressed enzyme possessed the high thermal stability characteristic of genuine PP1-Arch.

## DISCUSSION

### **Verification that PP1-Arch is a protein phosphatase**

The putative archaeobacterial protein phosphatase, PP1-Arch, was originally identified by J. S. Cantwell using an assay that measured the release of TCA-soluble radioactivity. Although it was initially assumed that this radioactivity was in the form of inorganic phosphate, it was also possible that it might be in the form of small TCA-soluble phosphopeptides generated by the action of a proteinase, rather than a protein phosphatase. In order to address this question I used two approaches. The first was a molybdate extraction of inorganic phosphate into organic solvents. During the extraction, phosphopeptides, if present, will remain in the aqueous phase. Both TCA and molybdate methods gave the same amount of  $^{32}\text{P}$  radioactivity (Table 2), indicating that the assay product of PP1-Arch was inorganic phosphate. The second approach was thin layer electrophoresis in which the assay product of PP1-arch was shown to comigrate with authentic [ $^{32}\text{P}$ ]phosphate on thin layer electrophoresis. These results demonstrated that PP1-Arch indeed was a protein phosphatase.

### **A single divalent metal ion-stimulated protein phosphatase was found in *Sulfolobus solfataricus***

A large number of serine/threonine-specific protein phosphatases have been identified in mammalian tissues. Among them, many are isoforms of the same type of serine/threonine protein phosphatase (Cohen *et al.*, 1990).

In *Sulfolobus solfataricus*, we believe there is only one divalent metal ion-stimulated protein phosphatase present inside the cells. There are several lines of evidence supporting it. First, Kennelly *et al* (1993) demonstrated that the addition of a second divalent metal ion failed to give any additional stimulation of PP1-Arch activity as compared to that observed when only  $Mn^{2+}$ , the most potent activator, was present at a saturating concentration. Second, from the activity profiles of DE-52, hydroxyapatite, G-100, and Mono Q FPLC chromatographic steps used in the purification of PP1-Arch, only one single peak of activity could be detected during any of my enzyme purifications. Using the gene of PP1-Arch as probe, there was only a single band detected in a Southern blotting of genomic DNA from *Sulfolobus solfataricus* (Figure 24). Whether there are other types of protein phosphatases in *Sulfolobus solfataricus* remains to be answered. However, from the sequence alignment results, PP1-Arch contains the same conserved motifs as several protein phosphatases from other organisms (Barton *et al*, 1994). It therefore is reasonable to speculate that degenerate probes derived from these regions should allow us to detect other forms of type 1/2A/2B protein phosphatases from *Sulfolobus solfataricus* or from any other archaeobacteria.

### **Purification of PP1-Arch**

PP1-Arch was the first protein phosphatase identified from any archaeobacterial species. This meant that there was no previous data to indicate whether it would show any homology with eukaryotic protein

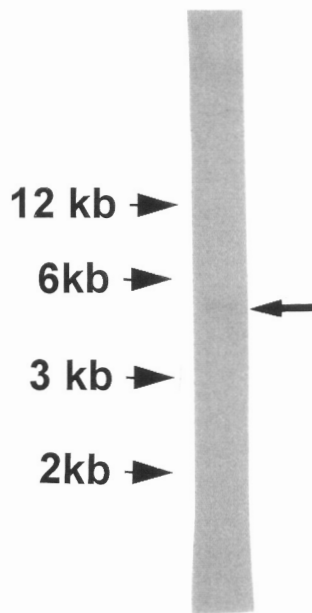


**Figure 24. Detection of a single PP1-Arch gene in *Sulfolobus solfataricus* genomic DNA by Southern Blotting.** Southern blotting was performed as described under "General procedures". Briefly, genomic DNA of *Sulfolobus solfataricus*, 5  $\mu$ g, was digested with *Cla*I. The mixture were loaded and analyzed by electrophoresis on a 1.0%(w/v) agarose gel. DNA fragments in the gel were denatured and transferred onto a nylon membrane. The membrane was hybridized with  $^{32}$ P-labeled DNA fragment corresponding to part of PP1-Arch gene ranging from amino acid #1 to amino acid #224. Shown is an autoradiogram of the hybridized membrane.

phosphatases. Therefore we decided that, instead of homology cloning, we would use protein purification and analysis of partial amino acid sequences as the initial steps toward cloning the PP1-Arch gene. A variety of techniques were tested for their efficiency in purifying PP1-Arch. Those eventually selected for use were ion exchange (CM, DE-52, and Mono Q), gel filtration (G-100), and hydroxyapatite chromatography. The following were tried and rejected for the reasons of low yield, poor resolution, or irreversible inhibition of PP1-Arch activity: ammonium sulfate precipitation, heat treatment, phosphocellulose, Blue Dextran, phenyl-Sepharose, and affinity (metal-chelation Sepharose including  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ni}^{2+}$ , thiophosphorylated myosin light chain-Sepharose 4B, casein-Sepharose 4B, histone-Sepharose 4B) chromatography. Overall, about a 1000-fold purification was achieved from the soluble fraction of *Sulfolobus solfataricus*. The final sample was nearly homogenous as determined by SDS-PAGE, which indicated that approximately 40% of the total protein present was PP1-Arch.

Since the final, Mono Q, fraction contained several polypeptides visible on an SDS-polyacrylamide gel, the unambiguous identification of the PP1-Arch protein became essential if we were to get the amino acid sequence data needed to clone its gene. The high temperature at which *Sulfolobus solfataricus* grows, as well as the results of our heat stability studies, suggested that PP1-Arch was quite stable and that its tertiary structure might resist denaturing agents to some extent. Hutchcroft *et al* (1988) had successfully renatured protein kinases *in situ* after SDS-PAGE. In an effort

to identify PP1-Arch from Mono Q fraction, I performed a similar renaturation experiment. My successful recovery of PP1-Arch activity permitted us to definitively identify and analyze the correct polypeptide.

Future purification experiments of PP1-Arch should be rendered easier as a result of the successful expression of PP1-Arch in *E.coli*. First, expressed PP1-Arch represents about 30% of the total protein of the *E.coli* cells (Figure 22), in contrast to less than 0.1% in the soluble fraction of *Sulfolobus solfataricus* (Table 3). However, at present, the majority of expressed PP1-Arch forms inclusion bodies. If we could renature the enzyme from these inclusion bodies, purifying large quantities of PP1-Arch to homogeneity should be very simple. If renaturation fails, prevention of the formation of inclusion bodies should be pursued. In addition, it should be possible to take advantage of some commercially available expression vectors in which His-tag or glutathione S-transferase fusion proteins are produced that can be easily purified by a metal-chelating or glutathione-sepharose column, respectively. Second, a heat step should be possible since PP1-Arch is extremely heat stable while the majority of *E.coli* proteins are not.

## **Characterization of PP1-Arch**

Serine/threonine protein phosphatases are characterized mainly on the basis of their molecular size, cellular location, substrate specificities, and their behaviors toward many protein phosphatase inhibitors or activators

(Shenolikar and Nairn, 1991).

### Molecular size and cellular location

The calculated molecular size of PP1-Arch based on the gene sequence, 33 kDa, agreed with that observed from SDS-PAGE. It also falls in the same range as the catalytic subunit of type 1/2A/2B protein phosphatases, 33 kDa - 35 kDa. The majority of PP1-Arch activity was found in the soluble fraction, even after ultracentrifugation. Computer analysis of the protein sequence does not reveal any significant hydrophobic domains, which also is consistent with the belief that PP1-Arch is located in the cytosol of *Sulfolobus solfataricus*.

### Activation by divalent metal ions

When PP1-Arch was purified in the presence of proteinase inhibitors, the activity of the final purified sample showed dramatic metal ion-dependence.  $Mn^{2+}$  was the best activator.  $Ni^{2+}$  and  $Co^{2+}$  also were effective in this role. Originally, divalent metal ion stimulation of the PP1-Arch activity and its insensitivity to okadaic acid led us to believe it might belong to the PP2C family. However, sequence data now have provided a completely definitive classification of PP1-Arch, which belongs to PP1/PP2A/PP2B superfamily.

Interestingly, on one occasion when PP1-Arch accidentally was purified in the absence of proteinase inhibitors, the Mono Q fraction showed 70% of activity in the absence of divalent metal ions as that observed in their

presence. These results suggest that the metal ion-binding site is located in a regulatory domain of the enzyme, and that somehow endogenous proteolysis caused the enzyme become metal ion-independent. However, subsequent attempts to activate PP1-Arch by partial proteolysis *in vitro* were unsuccessful, even when a 1:1 ratio of phosphatase to proteinase was used. This suggests the tertiary structure of the phosphatase is resistant to the actions of exogenous proteinases. In future, partially denatured phosphatase should be used for proteinase activation studies. Crystal structures of PP1-Arch in the presence or absence of divalent metal ions also should provide us with insights on the mechanism of divalent metal ion-activation.

### Inhibitors

Phosphate and pyrophosphate inhibit PP1-Arch, presumably via product inhibition. Their effects are reversible, as demonstrated by the recovery of activity from hydroxyapatite fraction after dialysis. Zinc and copper inhibition probably reflects high affinity binding to the divalent metal ion binding site, the result of which is to block enzyme activation by other metals. Since, so far, no tyrosine phosphatase activity has been detected towards a number of exogenous phosphotyrosine-containing substrates, and vanadate and molybdate, which are common tyrosine phosphatase inhibitors, had no effect on the archaeobacterial protein phosphatase, these results suggest that this enzyme is serine/threonine-specific. Okadaic acid, which is an inhibitor of eukaryotic PP1 and PP2A, also has no effect on our enzyme. This suggests that even though its DNA-derived sequence shows homology

with type 1 and 2A protein phosphatases from other organisms, there is no okadaic acid binding site in the archaeobacterial phosphatase sequence. A more distant homologue of PP1/PP2A, PP- $\lambda$  (Cohen et al., 1989), and the protein phosphatase activity in another archaeobacterium (Oxenrider and Kennelly, 1993), *Haloferax Volcanii*, were also insensitive to this compound. However, the protein phosphatase activity in a third archaeobacterium (Oxenrider et al., 1993), *Methanosaricina thermophila*, has been shown to be sensitive to okadaic acid.

Sulfhydryl-modifying reagents showed no effects on the catalytic activity of PP1-Arch. This result was very surprising, since it is known that PP1 and PP2A can be inactivated by sulfhydryl-modifying reagents (Shima et al., 1978; Nemani and Lee, 1993). However, Zhang et al. (1993) demonstrated that when all conserved histidine residues among PP1 and PP2A were mutagenized individually in rabbit muscle PP1, all of the mutants showed high catalytic activities. Together, these findings suggest that cysteine does not participate in the catalytic mechanism of the members of the PP1/PP2A/PP2B family. PP1-Arch was inactivated by diethylpyrocarbonate (DEPC), however. This agrees with the studies of Zhuo et al. (1994), in which they suggested that conserved histidine residues were important for the activity of PP- $\lambda$ . Among all type 1/2A/2B protein phosphatases, there are two conserved histidine residues, which correspond to His39 and His103 in PP1-Arch. It has been suggested that both PP1 and PP2A may share a common metal binding region in which the conserved histidine residues might be located (Vincent and Averill, 1990). All these

results suggest that histidine plays an essential role in catalysis. Site-directed mutagenesis of the two conserved histidine residues on PP1-Arch should prove it.

### Substrate Specificity

PP1-Arch is believed to be a serine/threonine-specific protein phosphatase, lacking any detectable activity toward phosphotyrosine proteins or peptides. It dephosphorylates a broad range of substrates *in vitro* including exogenous phosphoproteins such as casein, glycogen phosphorylase, phosphorylase kinase, histones, and myosin light chains. Studies with peptide substrates in the laboratory of Dr. L. Pinna revealed that the presence of basic residues on the N-terminal side of the phosphoserine/phosphothreonine in peptides is a positive determinant for PP1-Arch substrate recognition, while the presence of acidic residues has negative effects (unpublished results). So far, all substrates being tested are exogenous in nature. In order to define the physiological role of PP1-Arch in *Sulfolobus solfataricus*, finding its natural substrates is essential.

### **Cloning and sequencing of the PP1-Arch gene**

#### Construction of *Sulfolobus solfataricus* genomic DNA library

There are two reasons why we chose to generate a genomic DNA library from *Sulfolobus solfataricus* instead of a cDNA library. First, to date, very few introns had been found in genes of *Sulfolobus solfataricus* and these

were in genes for tRNAs, not proteins (Kain *et al.*, 1983). Second, many genes had been successfully cloned from *Sulfolobus solfataricus* using a genomic DNA library (Brown *et al.*, 1989). Under the assumption that large DNA fragments ( $\geq 10\text{kb}$ ) are less effectively ligated into a plasmid vector than small fragments, we used Southern blotting to test several different restriction enzymes for the purpose of generating a proper size range of DNA fragments for the construction of a genomic DNA library. *EcoRI* was initially chosen as the restriction enzyme for constructing a genomic DNA library from *Sulfolobus solfataricus*. The multiple bands hybridizing with oligonucleotide 1 shown on the autoradiogram (Figure 13) were probably a reflection of the low stringency hybridization and washing conditions used in this experiment.

Using Bacteriophage  $\lambda$ GEM-11 (Promega) as vector, a genomic DNA library of *Sulfolobus solfataricus* was also constructed. One advantage of this was that much fewer plaques needed to be screened, because it had a large insert, average 15 kb, in contrast to the average 2kb-5kb insert of the library in which plasmid was used as vector. However, the fact that plasmid was easier to handle than the bacteriophage caused us to stay with a plasmid system.

### "Touchdown" PCR

Molecular homology cloning is a technique that is often used to search for a new gene when its relatives share one or more conserved regions from which degenerate probes can be designed. Many protein kinases had been

successfully identified using homology cloning (Hanks and Linberg, 1991). Some of these protein kinase genes were initially isolated via the polymerase chain reaction (PCR) using degenerate oligonucleotides as primers. For PP1-Arch, we took advantage of the fact that we had determined two peptide sequences with known orientations to one another. We therefore decided to use PCR to amplify the region between these two peptides in order to generate a PCR product from the PP1-Arch gene. We planned to go on and use this DNA fragment as a probe to screen the library for the complete PP1-Arch gene.

Degenerate primers had successfully been used as PCR primers in many cases (Innis *et al.*, 1990). The key was to have a correct 3' end sequence. Based on peptide sequences information, three oligonucleotide primers were designed. In order to reduce the degeneracy of our primers, only A or T was chosen at some "wobble" positions since *Sulfolobus solfataricus* DNA is extremely AT-rich. Based on the assumption that those primers, even accounting for the degeneracies in oligonucleotides such as numbers 2, 3 and 4, should have the highest  $T_m$  with regard to the binding to the PP1-Arch gene, we decided to start the annealing at a temperature that was 5°C higher than the calculated  $T_m$  of oligonucleotide primers and gradually decrease it in the following cycles. This allowed the specific PCR product to be amplified many cycles before nonspecific products might appear. Our first round of "touchdown" PCR generated a single DNA fragment with a size of 560 bp. When the 560 bp fragment was amplified with a nested primer set, it generated a 520 bp fragment that was the same

size as predicted based on the known differences regarding where the N-terminal primers should bind the PP1-Arch gene. Later DNA sequence data proved that the 560 bp fragment indeed came from the gene for PP1-Arch. Therefore, we think "touchdown" PCR is particularly useful for cases when the  $T_m$  values of primers only can be roughly estimated, such as for degenerate oligonucleotides.

#### Screening of genomic DNA library of *Sulfolobus solfataricus*

Radiolabeled oligonucleotide probes are commonly used in screening for genes of interest. Using oligonucleotide 1 as probe, enough recombinant colonies were screened to insure a at least 99% probability that one of the recombinants should contain the PP1-Arch gene. It was shown that 3 out of the 25 "positive" colonies were genuinely positive. This result compares with experiments in which long DNA fragments were used as probes in which 5 out of 6 "positive" colonies genuinely positive. This indicates that false signals more frequently appear when degenerate oligonucleotides are used as probes. This problem can be minimized by replacing SSC with tetramethylammonium chloride (TMAC) in the hybridization solution (Ausubel *et al.*, 1987), and indeed I observed lower background levels when TMAC was used under the identical conditions as SSC. However, the fact that the SSC protocol required a 14 hour hybridization and the TMAC protocol requires 40 - 60 hours of hybridization caused me to use the former.

#### Sequence of the gene for PP1-Arch

PP1-Arch gene was sequenced on both strands using gene specific primers. The GC (33%) content of PP1-Arch matched the average GC content (36%) of the entire genome of *Sulfolobus solfataricus* (Brown *et al.*, 1989). GUG served as initiation codon for PP1-Arch.

Sequence comparison among protein phosphatases from eukaryotes, bacteriophage  $\lambda$ , and archaebacterium *Sulfolobus solfataricus* indicates there is about overall 30% sequence identity between PP1-Arch and PP1/PP2A/P2B. This clearly reveals that PP1-Arch belongs to the type 1/2A/2B protein phosphatase family. There are three highly conserved motifs in the NH<sub>2</sub>-terminal region of all type 1/2A/2B protein phosphatases (Barton *et al.*, 1994; Zhuo *et al.*, 1994), which also presents in the sequence of PP1-Arch. Their sequences are GD(TIVL)HG, GD(YL)VDR, and (RV)GNHE. This homology suggests that protein phosphatases are highly conserved through evolution, and must play an important role essential for cells to survive during evolution.

Phylogenetically, PP1-Arch is closer than PP- $\lambda$  to eukaryotic protein phosphatases. This agrees with other biochemical and molecular biological data, which suggests that archaebacteria are closer to eukaryotes than are eubacteria. The phylogenetic tree also suggests that there is a common ancestor between PP1-Arch and eukaryotic protein phosphatases.

### **Expression of PP1-Arch**

pT7-7 is a very commonly used expression vector, primarily because

of the high specificity and high rate of T7 RNA polymerase (Tabor and Richardson, 1985). But when the expression of T7 RNA polymerase is not under tight control and the gene product of interest is toxic to the host cells, this imposes a boundary. Expression of PP1-Arch was toxic to *E.coli* host strain BL21(DE3) grown on LB medium, even when the expression of T7 RNA polymerase was not induced with IPTG. Fortunately, the toxicity was mild when the cells were grown on M9<sup>amp</sup> medium, suggesting that there are some sugar molecules in LB medium that induce the expression of PP1-Arch. I think fresh transformants should be used in the future for large scale expression of PP1-Arch, because the mild toxicity of PP1-Arch to host strains of *E.coli* could make the cells lose the ability to express PP1-Arch after several rounds of usage.

The complete elimination of 'leaky' basal expression of PP1-Arch could be achieved by introducing T7 RNA polymerase gene through timed transfection using bacteriophage CE6 (Studier and Moffatt, 1986) at the same time as the inducer IPTG is added.

The majority of expressed PP1-Arch formed inclusion bodies. This problem may be overcome by growing the cells at lower temperature (Schein and Noteborn, 1988), in different media (Ausubel, 1987), or using a different host strains or expression vectors (Chang *et al.*, 1978),

### **Areas to be exploited in the future**

The next questions we should ask are what is the endogenous substrate

of PP1-Arch and what is the physiological role of PP1-Arch. In *Sulfolobus solfataricus*, there were only a few [<sup>32</sup>P]phosphoproteins that can be detected on SDS-PAGE (Skorko, 1984; Zylka, 1994). If the gel were treated with PP1-Arch, any band disappearing from the autoradiogram must be a substrate for PP1-Arch, and phosphopeptide sequence analysis could provide a tool for cloning the endogenous substrate.

In terms of understanding the catalysis, substrate binding, metal ion activation, and other functions for PP1-Arch, site directed mutagenesis experiments could be conducted. PCR can be used as a quick way of doing that (Ho *et al.*, 1989). Large scale expression of PP1-Arch will facilitate obtaining enough protein for crystallization of PP1-Arch, and the crystal structure certainly will provide useful information to address those questions.

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## ABSTRACTS

P.J. Kennelly, P. Marchand and J. Leng, (1991) "5'-p-Fluorosulfonyl Benzoyl Adenosine as a probe of the MgATP Binding Site of Myosin Light Chain Kinase. Comparison of the Calmodulin-Bound and Calmodulin-Free Enzyme", *FASEB Journal* 5, A449.

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