# A thermostable vacuolar-type membrane pyrophosphatase from the archaeon *Pyrobaculum aerophilum*: implications for the origins of pyrophosphate-energized pumps

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**Abstract Vacuolar-type $H^+$-translocating pyrophosphatases (V-PPases) have been considered to be restricted to plants, a few species of phototrophic proteobacteria and protists. Here, we describe PVP, a thermostable, sequence-divergent V-PPase from the facultatively aerobic hyperthermophilic archaeon *Pyrobaculum aerophilum*. PVP shares only 38% sequence identity with both the prototypical V-PPase from *Arabidopsis thaliana* and the $H^+$-PPi synthase from *Rhodospirillum rubrum*, yet possesses most of the structural features characteristic of V-PPases. Heterologous expression of PVP in *Saccharomyces cerevisiae* yields a $M_r$ 64 000 membrane polypeptide that specifically catalyzes $Mg^{2+}$-dependent PPi hydrolysis. The existence of PVP implies that PPi-energized $H^+$-translocation is phylogenetically more deeply rooted than previously thought.**



*Key words:* Archaeon; Inorganic pyrophosphatase; Proton pump; Pyrophosphate; Vacuolar-type $H^+$-pyrophosphatase; *Pyrobaculum aerophilum*

## 1. Introduction

Inorganic pyrophosphatases (PPases; EC 3.6.1.1) fall into two major classes, soluble PPases and membrane-associated $H^+$-translocating PPases ($H^+$-PPases). Both categories are thought to control intracellular pyrophosphate (PPi) levels and confer a favorable poise on otherwise thermodynamically unfavorable pyrophosphorylytic biosynthetic reactions but in different ways. Whereas soluble PPases simply hydrolyze PPi dissipatively to yield heat [1], membrane-associated $H^+$-PPases conserve some of the energy liberated during PPi hydrolysis as transmembrane $H^+$ gradients [2].

The most thoroughly investigated membrane-associated $H^+$-PPases are those found on the vacuolar membrane of plant cells and the energy-coupling membranes of the phototrophic, purple, non-sulfur proteobacterium *Rhodospirillum rubrum* [3–5]. Plant vacuolar $H^+$-PPases have a near obligate requirement for mM concentrations of $K^+$ for activity, appear to operate exclusively in a hydrolytic mode, pumping $H^+$ from the cytosol into the vacuole at the expense of PPi, and are considered to contribute to the generation of the transtonoplast $H^+$-electrochemical potential difference required for the secondary transport of a broad range of solutes [3]. Phototrophic bacterial $H^+$-PPases are, by comparison, relatively insensitive to monovalent inorganic cations, freely reversible and considered to contribute to the maintenance of $H^+$ gradients when irradiance is insufficient to sustain direct $H^+$-coupled ATP synthesis, through the utilization of photosynthetically derived cellular PPi reserves [5,6].

As substantiated by the recent molecular cloning of a homolog of the prototypical plant vacuolar $H^+$-PPase, *Arabidopsis thaliana* vacuolar pyrophosphatase (AVP) [7], from *R. rubrum* (RVP) [8], the plant and phototrophic bacterial enzymes belong to the same category of $H^+$-phosphohydrolases, a fourth category distinct from F-, P- and V-ATPases [2]. Constituted of a single 75–81 kDa intrinsic membrane protein species, which alone is sufficient for PPi, strictly dimagnesium pyrophosphate ($Mg_2PPi$), hydrolysis and $H^+$-translocation [4], the subunit composition of vacuolar-type $H^+$-PPases (V-PPases[1]) is rudimentary by comparison with that of their V- and F-ATPase counterparts.

Notwithstanding their simple organization, abundance and high catalytic potential in plants and phototrophic bacteria [3,5], a major impediment to understanding the basic biology and mechanism of action of V-PPases has been the lack of sequence data from organisms other than plants. On the one hand, the occurrence of V-PPases in plants, their algal antecedents and the purple α-proteobacteria, and their apparent absence from animals and fungi, has given rise to the notion that plant V-PPases may have arisen by lateral gene transfer from an α-proteobacterial endosymbiont [3]. On the other hand, the lack of sequence-divergent homologs, the fact that, until the molecular cloning of RVP, all published sequences were from the same group of organisms, plants, and exhibited amino acid sequence identities of 80% or greater [4], has severely impeded attempts to identify conserved amino acid residues of potential mechanistic significance on a rational basis. Despite recent reports of partial V-PPase-like coding sequences in the thermophilic bacterium *Thermotoga maritima* [9] and the marine alga *Acetabularia mediterranea* [10] and of V-PPase-like activities or immunoreactive polypeptides in the

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*Abbreviations:* AMDP, aminomethylenediphosphonate; AVP, *Arabidopsis thaliana* vacuolar $H^+$-pyrophosphatase; NEM, *N*-ethylmaleimide; DCCD, *N,N′*-dicyclohexylcarbodiimide; PVP, *Pyrobaculum aerophilum* vacuolar-type pyrophosphatase; RVP, *Rhodospirillum rubrum* vacuolar-type $H^+$-pyrophosphatase; V-PPase, vacuolar-type $H^+$-pyrophosphatase

[1] Herein, the term 'V-PPase' is applied to any membrane-associated PPase exhibiting systematic homology to the prototypical vacuolar $H^+$-PPase, AVP.


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parasitic protist *Trypanosoma cruzi* [11], the syntrophic bacterium *Syntrophus gentianae* [12] and a broad range of cyanobacteria [13], which are in themselves indicative of a pump whose occurrence is not restricted to plants and other phototrophs, no studies have been published describing the complete sequence of a V-PPase from a non-photosynthetic organism or the functional characterization of a V-PPase clone from a non-plant source.

In this communication, we describe the isolation, sequence analysis and functional characterization of PVP, a sequence-divergent V-PPase homolog from a facultatively aerobic, hyperthermophilic archaeon, *Pyrobaculum aerophilum*. The results of these studies unequivocally demonstrate a bona fide V-PPase ortholog in a phylogenetically ancient non-photosynthetic organism, disclose a membrane-associated PPi-energized enzyme of extraordinary thermostability and yield fresh insights into the identity of sequence motifs likely involved in core catalysis by all V-PPases. A corollary of these investigations, verified by the results of genomic database searches deploying class-specific sequences identified through sequence comparisons between PVP, RVP and plant V-PPases, is that these pumps are much more widely distributed, sequence-divergent and ancient than was suspected before.

## 2. Materials and methods

### 2.1. Isolation of PVP

The 2.3 kb sequence of an open reading frame (ORF) identified during the course of the *P. aerophilum* genome sequencing project [14] as encoding a hypothetical protein resembling a plant V-PPase was amplified from BAC clones 7C5 and 3F9 by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene) and unique primers corresponding to positions 1–20 and 2297–2316 of the coding sequence of the candidate gene. After establishing their identity by sequencing, the PCR products derived from the BAC clones were restricted with *Not*I and *Sal*I and subcloned into yeast *Escherichia coli* shuttle vector pYES3 [15] to generate pYES3-PVP.

### 2.2. Heterologous expression in yeast

To enable comparisons between PVP and AVP after heterologous expression in *Saccharomyces cerevisiae*, the coding sequence of *PVP* was PCR-amplified from pYES3-PVP and subcloned into expression vector pYES2 (Invitrogen). For this purpose, an oligonucleotide corresponding to the *PVP*-flanking *CYC1* termination region of pYES3 and another corresponding to positions 1–20 of the coding sequence of *PVP*, in which the putative *P. aerophilum* initiator codon, GTG, was replaced by a yeast-compatible ATG codon, were used as primers. After digestion with *Xba*I, the 2.1 kb PCR product was ligated into the multicloning site of *Pvu*II-*Xba*I double-digested pYES2 to yield construct pYES2-PVP, containing the entire coding sequence of *PVP* inserted between the yeast *GAL1* promoter and *CYC1* termination sequences of the plasmid. Plasmid pYES2-AVP, containing the entire coding sequence of *AVP* was constructed as described [16].

Vacuolar protease-deficient *S. cerevisiae* strain BJ5459 (*Matα, ura3-52, trp1, lys2-801, leu2Δ1, his3-Δ200, pep4::HIS3, prbΔ1.6R, can1, GAL*) transformants containing empty vector (pYES2), pYES2-PVP or pYES2-AVP were generated by the LiOAc/PEG method [17], selected for uracil prototrophy and subjected to membrane fractionation as described [16,18].

### 2.3. Preparation of membranes from P. aerophilum

One l of stationary phase cultures of *P. aerophilum* (type strain IM2; DSMZ7523), grown aerobically as described [19], were pelleted by centrifugation at 5500×*g* for 25 min, washed twice in 50 mM MOPS buffer (pH 7.0) and disrupted by two passages through a French press at 18 000 psi. Unbroken cells and debris were pelleted by centrifugation at 8000×*g* for 10 min and membranes were recovered from the low-speed supernatant by centrifugation at 100 000 for 1 h. The membranes were washed twice and resuspended in 5 mM Tris-HCl buffer (pH 8.0) containing 0.4 M glycerol and 1 mM Tris-EGTA and used immediately or stored at −85°C.

### 2.4. PPase activity assays, Western analyses and protein estimations

PPase activity was assayed as described [4], except that imidazole-based, rather than Tris- or Bis-Tris-propane-based, buffers were used throughout to preclude competition with $K^+$ and other monovalent cations [20]. Unless otherwise indicated, PVP and AVP-mediated PPase activity was assayed at 75 and 37°C, respectively. Activities were expressed as μmol PPi hydrolyzed/mg protein/min. The susceptibility of PVP and AVP to irreversible inhibition by *N*-ethylmaleimide (NEM) and *N,N′*-dicyclohexylcarbodiimide (DCCD) was determined as described [18,21]. For Western analyses of endogenous and heterologously expressed PVP and heterologously expressed AVP, membrane samples were subjected to denaturation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransfer and immunoreaction with antibodies $PAB_{HK}$ or $PAB_{TK}$, as described [18]. Immunoreactive bands were visualized by ECL (Amersham Pharmacia Life Sciences). Protein was estimated by the method of Bradford [22].

### 2.5. Computer programs

The putative membrane topology of PVP was modeled using TopPred II, version 1.3 [23] as detailed for AVP [18]. V-PPase-like sequences in other organisms were identified by BLAST searches of preliminary sequence data collated by The Institute for Genomic Research (http://www.tigr.org).

## 3. Results

### 3.1. Isolation of PVP

The partial sequence of a gene capable of encoding a hypothetical plant V-PPase homolog was found during the course of sequencing the genome of *P. aerophilum* [14]. PCR amplification of this 2166 bp sequence from two overlapping *P. aerophilum* BAC clones, both of which encompassed it entirely, revealed a complete ORF for a 722 amino acid (75 kDa) polypeptide (Fig. 1). Because of the overall sequence identity (38%) and similarity (58%) of its deduced translation product to both AVP [7] and RVP [8][2], the *P. aerophilum* ORF was tentatively designated *PVP*.

A minor uncertainty concerning the precise location of the initiator codon of *PVP*, whether it was a Val (GTG) codon at position 1 or a more conventional Met (ATG) codon at position 7, was not pursued further because it neither markedly affected the predicted size of the polypeptide nor its alignment with other V-PPases. The longer of the two possible deduced polypeptides is depicted in Fig. 1.

### 3.2. Sequence characteristics

Several features of the polypeptide encoded by PVP were consistent with it being a sequence-divergent V-PPase homolog. Parallel application of hydrophobic moment analysis [24], the positive-inside rule [25] and the charge-difference rule [26] using the TopPred II program [23] predicted equivalent structures containing 15 or 16 transmembrane spans for both PVP and AVP (Fig. 1). Of the two top-ranked models for PVP and AVP, the 15-span model presented in Fig. 1 was favored over the 16-span model because it not only satisfied

[2] From hereon, AVP is used as a model for all plant V-PPases because all share greater than 80% sequence identity, making any one representative of the others. Since the functional capabilities of the RVP clone have not yet been determined [8], the characteristics of PVP are compared with those of AVP, which has been functionally characterized [16,18,21].

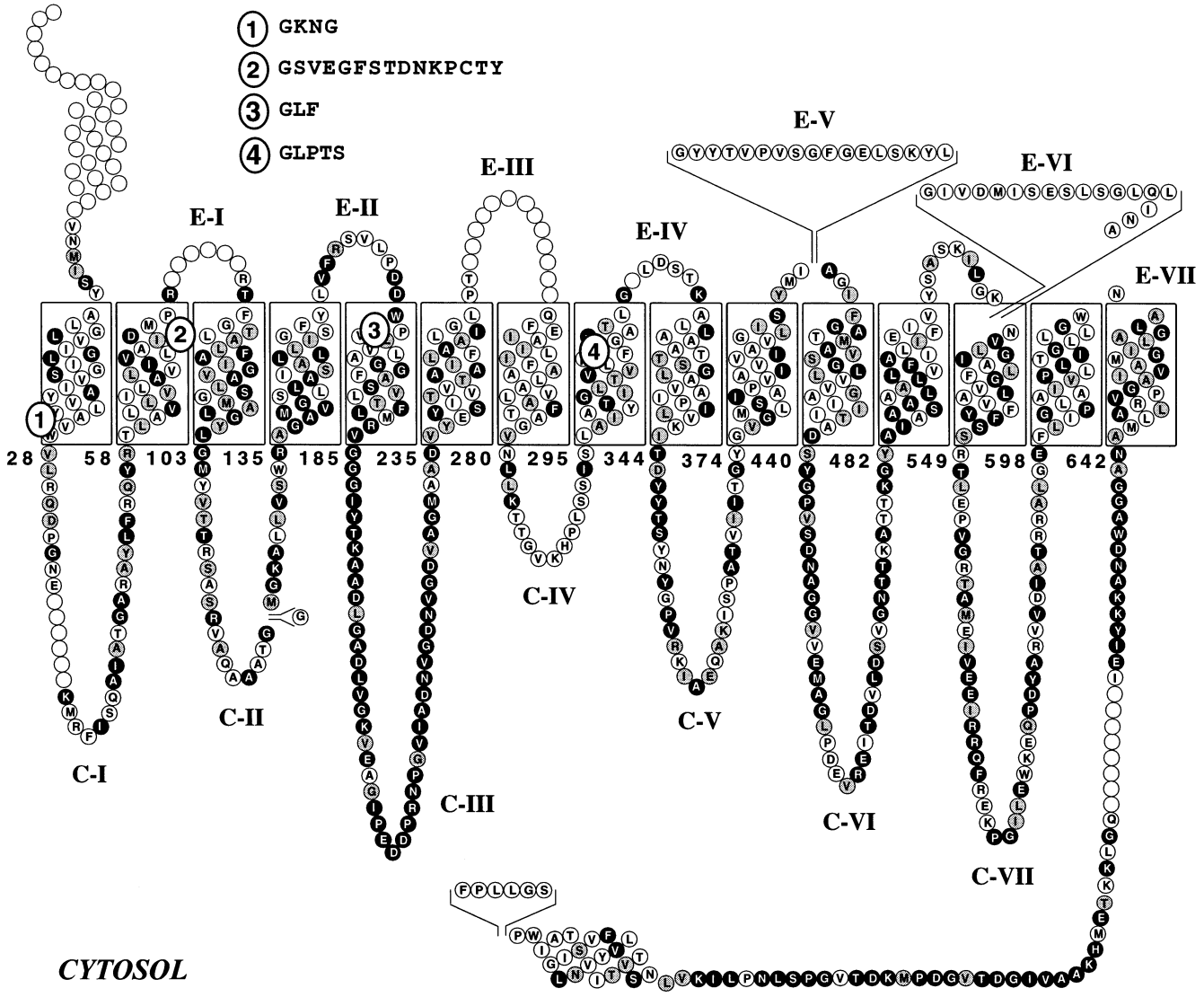


Fig. 1. Deduced amino acid sequence, predicted topology and alignment of PVP with AVP. Residues identical between PVP and AVP are shown in white on a black background, similar residues are shown in black on a gray background. Amino acid residues within AVP missing from PVP are denoted by open circles or, when they occur in putative transmembrane spans, as numbers, the sequences corresponding to the latter are shown above the main figure. Stretches of sequence unique to PVP are indicated as insertions. Topology was predicted using TopPred II [23]. The GenBank accession number for PVP is AF182812.

the criteria applied by TopPred II but also predicted a cytosolic oriented C-terminus. A 16-span model, necessitating an extracytosolic C-terminus, was incompatible with the finding that fusion of apoaequorin with the C-terminus of AVP generates a vacuolar membrane-localized polypeptide capable of sensing cytosolic free $Ca^{2+}$ in transgenic *Arabidopsis* plants [27].

As would be predicted for a polytopic membrane protein requiring high stringency interactions with cytosolic ligands such as substrate ($Mg_2PPi$), activator ($Mg^{2+}$) and inhibitor ($Ca^{2+}$) (reviewed in [4]), the sequences exhibiting the greatest similarity between PVP and AVP were those located in putative hydrophilic loops with a cytosolic disposition. The aggregate sequence similarity between the cytosolic loops of PVP and AVP was 65% by comparison with only 25% for the extracytosolic loops. Except for short 6 and 8 amino acid residue deletions from cytosolic loop I and the C-terminal tail, respectively, and a single (Gly) residue insertion at position 125 in cytosolic loop II in PVP (Fig. 1), sequence contiguity between PVP and AVP was conserved throughout the cytosolic domains. Of the eight cytosolic sequence segments, loop III and the C-terminal tail were the most sequence-conserved between PVP and AVP: 97 and 77% similar, respectively. By contrast, alignment contiguity of the extracytosolic hydrophilic loops was interrupted at several sites by lengthy sequences that were either missing from or inserted into one or other of the two polypeptides. The first 30 N-terminal amino acid residues of AVP, which encompass the first transmembrane span of this sequence, were missing from PVP, though topological equivalence between PVP and AVP was maintained because residues 7–27 of the former satisfied the requirements of a transmembrane α-helix (Fig. 1). Extracytosolic loops I, III and IV of PVP were missing six, 13 and one residues, respectively, whereas extracytosolic loops V and VI contained 17 and 18 amino acid residue insertions, respectively, versus AVP (Fig. 1). The only exception to this pattern

was the C-terminal tail which was extended by six additional residues in PVP (Fig. 1).

PVP contained many of the sequence motifs speculated or demonstrated to be necessary for catalysis by plant V-PPases. The most sequence-conserved loop of PVP, cytosolic loop III, contained the putative 'catalytic' motif, $DX_7KXE$, found in both soluble and membrane-associated PPases [2]. PVP cytosolic loop V contained the span-loop interface motif TDYYTS which in AVP occurs in the form TEYYTS and encompasses $Glu^{427}$, a residue implicated in coupling PPi hydrolysis to $H^+$-translocation [18]. Cytosolic loop VI of PVP contained the motif DSYGP, containing one of the two acidic residues ($Asp^{504}$), which in AVP contributes to the susceptibility of the pump to inhibition by DCCD [18]. Of strategic value for the visualization of endogenously and heterologously expressed PVP, both of the sequences recognized by the V-PPase-specific antibodies, $PAB_{TK}$ and $PAB_{HK}$ [16], TKAADVGADLVGKIE and HKAAVIGDTIGDPLK, respectively (the former of which encompassed the $DX_7KXE$ motif), were conserved in cytosolic loop III and the C-terminal tail of PVP, except for two conservative (Val to Leu, Ile to Val) substitutions in the first motif and two (Ile to Val, Leu to Met) in the second.

Two residues present in AVP that were absent from PVP were $Cys^{634}$ of cytosolic loop VII and the second DCCD-binding residue, $Glu^{305}$, of putative transmembrane span VI. AVP $Cys^{634}$ is essential for inhibition of the enzyme by maleimides but not for catalysis [21]. AVP $Glu^{305}$ has the properties of a residue that contributes to both catalytic activity and DCCD inhibitability [18].

### 3.3. Heterologous expression of PVP

To determine if its structural resemblance to AVP and other V-PPases had a functional basis and if PVP alone is sufficient for catalysis, the activity of heterologously expressed PVP was investigated. For this purpose, the putative GTG start codon of *PVP* was substituted with a yeast-compatible ATG codon and *S. cerevisiae* strain BJ5459 was transformed with empty vector (pYES2) or vector containing the entire ORF of *PVP* (pYES2-PVP). For comparisons with AVP under identical conditions, the same yeast strain was transformed with pYES2-AVP.

High levels of PVP expression were achieved by this approach. Vacuolar membrane-enriched vesicles purified from pYES2-PVP-transformed *S. cerevisiae* strain BJ5459 (BJ5459/pYES2-PVP) cells contained an intense $PAB_{HK}$- (and $PAB_{TK}$) reactive $M_r$ 64 000 band which was absent from the corresponding fraction from untransformed or empty vector control BJ5459 cells (Fig. 2). Moreover, PVP appeared to be expressed and undergo membrane insertion at a similar efficiency to AVP in this heterologous system. The $M_r$ 64 000 and $M_r$ 67 000 polypeptides in the vacuolar membrane-enriched fractions from BJ5459/pYES2-PVP and BJ5459/pYES2-AVP cells, respectively, reacted with antibody $PAB_{HK}$ with similar intensities (Fig. 2). As noted consistently in previous investigations of endogenous and heterologous V-PPases and attributed to anomalous electrophoretic properties associated with their extreme hydrophobicity [4], both PVP and AVP migrated at lower $M_r$ values than would be predicted from their computed masses.

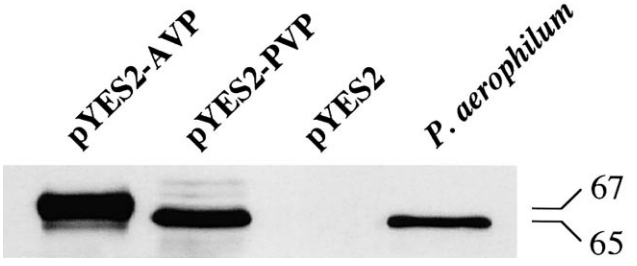


Fig. 2. Western analysis of antibody $PAB_{HK}$-reactive polypeptides in vacuolar membrane-enriched vesicles purified from pYES2-AVP, pYES2-PVP and pYES2-transformed *S. cerevisiae* BJ5459 cells and membranes from *P. aerophilum*. Protein (5 μg) was subjected to SDS-PAGE on 10% (w/v) gels, electrotransferred and probed with antibody $PAB_{HK}$. The bands shown were the only immunoreactive species detected. Blots probed with antibody $PAB_{TK}$ yielded the same results.

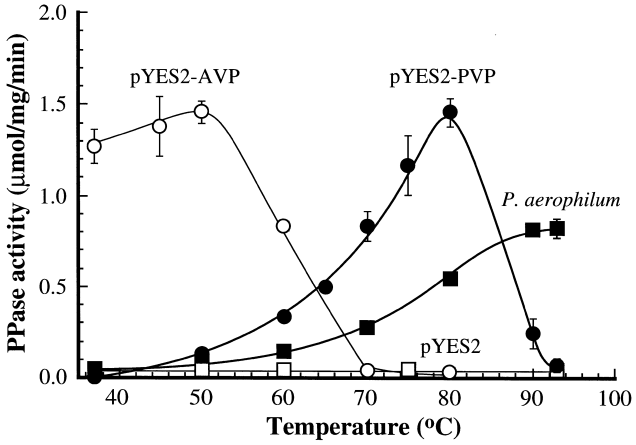


Fig. 3. Temperature-dependence of PVP- and AVP-mediated PPi hydrolysis. PPi hydrolysis by vacuolar membrane-enriched vesicles purified from pYES2-PVP, pYES2-AVP and pYES2-transformed *S. cerevisiae* BJ5459 cells and membranes from *P. aerophilum* was measured at the temperatures indicated after pre-incubation of the membranes (1–3 μg protein) for 10 min at the desired temperature in assay medium (300 μl) minus PPi before the initiation of reaction by the addition of imidazole-PPi (0.3 mM). Values shown are means ± S.E.M. ($n = 3$).

### 3.4. PVP-mediated PPi hydrolysis

The $PAB_{HK}$-reactive polypeptide detected in BJ5459/pYES2-PVP cells was competent in PPi hydrolysis. When assayed in reaction buffer containing 250 μM NaF to abolish any contribution from contaminating endogenous yeast soluble PPase [16], PVP-mediated PPi hydrolysis was appreciable. However, while establishing orthology between PVP and AVP, in terms of the basic equivalence of their phosphohydrolase activities, these experiments also revealed several marked differences between the two enzymes. Most striking was the higher temperature optimum and thermal stability of PVP. Whereas AVP-catalyzed PPi hydrolysis was optimal at 40–50°C, achieving a maximum of 1.2–1.4 μmol PPi/mg/min, appreciable PVP-catalyzed PPi hydrolysis required temperatures in excess of 70°C (optimum 80°C, maximal activity 1.5 μmol PPi/mg/min) (Fig. 3). Likewise, BJ5459/pYES2-PVP membranes retained more than 95% of their fluoride-insensitive PPase activity after incubation for 2 h at 75°C, whereas more than 90% of the corresponding activity of BJ5459/pYES2-AVP membranes was abolished within 4 min

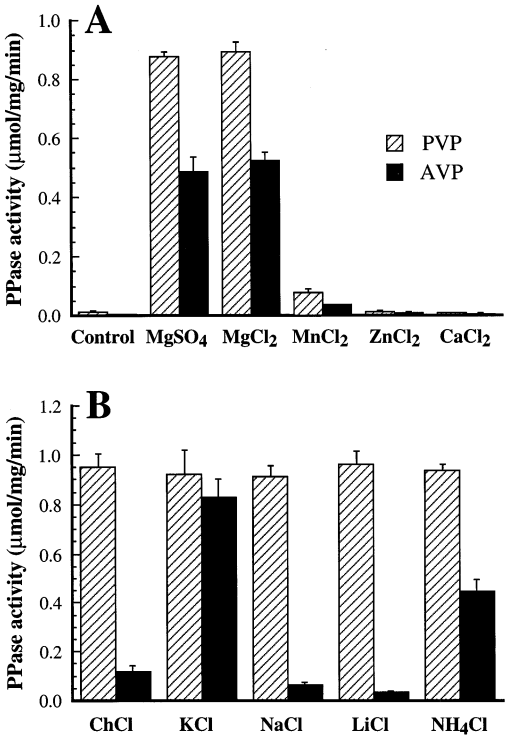


Fig. 4. Inorganic cation-dependence of PPi hydrolysis by PVP and AVP. A: Divalent cations. B: Monovalent cations. PPi hydrolysis by vacuolar membrane-enriched vesicles (3–5 μg membrane protein) purified from pYES2-PVP and pYES2-AVP-transformed *S. cerevisiae* BJ5459 cells was measured in reaction media (300 μl) containing 1.3 mM concentrations of the divalent cations indicated plus 100 mM KCl (A) or 100 mM concentrations of the monovalent cations indicated plus 1.3 mM $MgSO_4$ (B). PVP-dependent and AVP-dependent PPi hydrolysis were measured at 75 and 37°C, respectively. Values shown are means ± S.E.M. ($n = 3$).

of incubation at the same temperature (data not shown). Since vacuolar membrane-enriched vesicles purified from BJ5459/pYES2 cells contained negligible fluoride-insensitive PPase activity, regardless of the assay conditions employed (Fig. 3A), the activities measured in the corresponding fractions from BJ5459/pYES2-PVP and BJ5459/pYES2-AVP cells were attributed exclusively to PVP and AVP, respectively.

### 3.5. Other phosphoanhydrides

The phosphohydrolase activities of PVP and AVP were directed primarily towards PPi. Although both were able to hydrolyze tripolyphosphate (PPPi), albeit at only 18 and 10% (0.45 and 0.08 μmol/mg/min) of the rates determined for the same concentration of PPi (0.3 mM), neither PVP nor AVP catalyzed the hydrolysis of ATP, ADP, AMP or PEP (data not shown).

### 3.6. Cation requirements

PVP and AVP shared an obligate requirement for $Mg^{2+}$ for activity but were differentially responsive to monovalent cations. Omission of $Mg^{2+}$ from the reaction medium abolished both PVP- and AVP-mediated PPi hydrolysis (Fig. 4A) and in both cases, $Mn^{2+}$, but not $Zn^{2+}$ and $Ca^{2+}$, could partially (50–60%) substitute for $Mg^{2+}$ (Fig. 4A). Unlike AVP, whose activity was increased by 7- and 4-fold by inclusion in the assay medium of 100 mM KCl or $NH_4Cl$, respectively, PVP was no more activated by $K^+$ and the other inorganic monovalent cations tested than by the non-stimulatory organic cation, choline (Fig. 4B).

### 3.7. Inhibitor sensitivity

Both PVP and AVP were sensitive to inhibition by DCCD but differentially sensitive to inhibition by NEM and aminomethylenediphosphonate (AMDP) (Table 1). Despite its lack of one of the two acidic residues ($Glu^{305}$), which in AVP is implicated in DCCD-binding and irreversible inhibition by this hydrophobic carbodiimide [18], PVP was slightly more sensitive to inhibition by this compound than AVP (Table 1). As would be expected from the absence of an equivalent of AVP $Cys^{634}$ from PVP and in agreement with the results from site-directed mutagenesis experiments demonstrating that AVP C634S or C634A substitutions render the enzyme maleimide-insensitive while leaving catalytic activity unaffected [21], PVP was not susceptible to MgPPi-protectable inhibition by NEM (Table 1). Whereas 20 μM concentrations of AMDP, a potent inhibitor of plant, protist and phototrophic bacterial V-PPases [4,11], diminished the hydrolytic activity of AVP by more than 75%, 100 μM concentrations diminished the activity of PVP by only 25% (Table 1).

### 3.8. Endogenous PVP

The applicability of the results obtained with heterologously expressed PVP to the endogenous archaeal enzyme was verified by Western analyses and activity measurements on membranes prepared from aerobic cultures of *P. aerophilum*. Membranes from *P. aerophilum* cultures not only contained a $M_r$ 64 000 polypeptide that co-migrated with heterologously expressed PVP and reacted intensely with antibody $PAB_{HK}$ (and $PAB_{TK}$) after SDS-PAGE and Western analysis (Fig. 2) but also appreciable fluoride-insensitive PPase activity (Fig. 3). The maximal activity of the endogenous enzyme (0.8 μmol PPi/mg/min), which was attained at a temperature coincident with that optimal for the growth of *P. aerophilum* (90–95°C) [19], was approximately half that of the heterologously expressed enzyme.

Table 1
Sensitivities of PVP and AVP to inhibition by AMDP, NEM and DCCD

| Agent | PPase activity (μmol PPi/mg/min) | |
|---|---|---|
| | pYES2-PVP | pYES2-AVP |
| AMDP (μM) | | |
| 0 | 1.26 ± 0.08 (100) | 0.88 ± 0.02 (100) |
| 1 | 1.26 ± 0.01 (100) | 0.72 ± 0.02 (82) |
| 20 | 0.88 ± 0.04 (70) | 0.22 ± 0.01 (25) |
| 100 | 0.94 ± 0.02 (75) | 0.18 ± 0.03 (20) |
| NEM (μM) | | |
| 0 | 1.05 ± 0.01 (100) | 1.47 ± 0.003 (100) |
| 100 | 1.02 ± 0.01 (100) | 0.09 ± 0.06 (6) |
| 100+MgPPi | 1.06 ± 0.03 (100) | 0.74 ± 0.04 (50) |
| DCCD (μM) | | |
| 0 | 1.12 ± 0.08 (100) | 0.78 ± 0.01 (100) |
| 50 | 0.30 ± 0.01 (27) | 0.41 ± 0.01 (53) |
| 100 | 0.16 ± 0.01 (14) | 0.22 ± 0.01 (28) |
| 200 | 0.13 ± 0.01 (12) | 0.14 ± 0.02 (18) |

The PPi hydrolytic activities of PVP and AVP were assayed in vacuolar membrane-enriched vesicles purified from *S. cerevisiae* BJ5459/pYES2-PVP and BJ5459/pYES2-AVP cells, respectively. Values in parentheses are activities expressed as % of control. 'MgPPi' = 1.3 mM $MgSO_4$ + 0.3 mM imidazole-PPi. Values shown are means ± S.E.M. ($n = 4$).

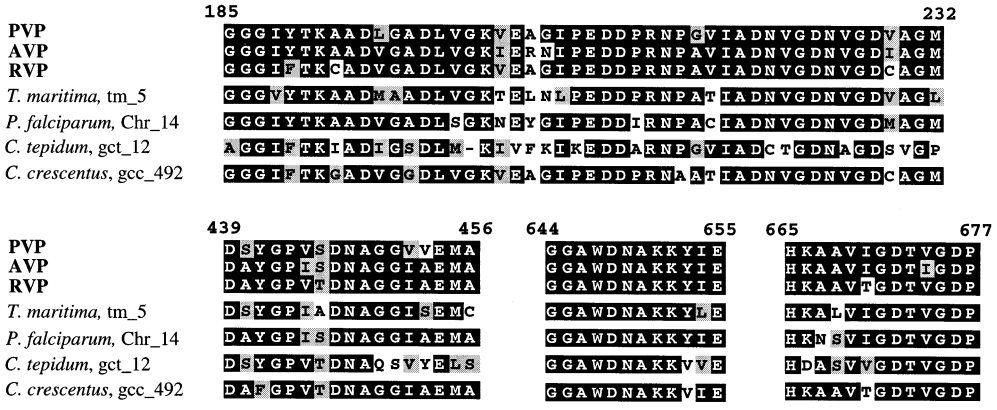


Fig. 5. Alignment of sequences exhibiting greatest conservation among PVP, AVP and RVP and the corresponding sequences of hypothetical V-PPase-like gene products identified by BLAST searches of genomic sequence databases. The sequences shown, in addition to those of PVP, AVP and RVP, are from *T. maritima* (contig tm_5; gi|2738605), *P. falciparum* (chromosome 14), *C. tepidum* (contig gct_12) and *C. crescentus* (contig gcc_492). Identical residues are shown in white on a black background. Similar residues are shown in black on a gray background.

### *3.9. A note on PVP-mediated $H^+$-translocation*

The high temperature optimum of PVP precluded direct measurements of its capacity for $H^+$-translocation. Neither yeast vacuolar membrane-enriched vesicles containing heterologously expressed PVP nor *P. aerophilum* membranes containing endogenous PVP were capable of establishing pH gradients when incubated at the elevated temperatures required for PVP-mediated PPi hydrolysis ($>55$°C). In the case of the membrane fractions from *P. aerophilum*, incomplete vesiculation was probably the cause in that these membranes could not sustain artificially imposed inside acid pH gradients at any of the temperatures examined (25–80°C). In the case of the yeast vacuolar membrane-enriched vesicles, the high passive conductance of the membranes to $H^+$ at the temperatures required for PVP-mediated PPi hydrolysis was probably the cause in that the same vesicles containing heterologously expressed AVP were able to achieve high rates and extents of PPi-dependent intravesicular acidification at 30°C [18] but not at 40–50°C, despite the slight elevation of PPi hydrolytic activity between 30 and 40°C (Fig. 3).

Purification and reconstitution of PVP into liposomes containing, for example, archaeal tetraether lipids was considered but rejected because, to our knowledge, in all cases in which the reconstitution of archaeal integral membrane proteins has been accomplished, ionic gradients were not sustainable at the temperatures required for PVP activity [28]. Analogously, while it has been suggested that $Na^+$, rather than $H^+$, is the sole energy-coupling ion in some thermophiles because background $Na^+$ permeability is generally lower than $H^+$ permeability [29], this possibility seemed remote for PVP in that PPi hydrolysis by this enzyme did not behave as if it was obligatorily coupled to $Na^+$ transport: PVP was completely insensitive to $Na^+$ and other inorganic monovalent cations (Fig. 4B).

### *3.10. Other V-PPases*

Having defined the basic structural and functional equivalence of PVP and AVP, the sequences of these two polypeptides plus that of the only other known sequence-divergent V-PPase, RVP, were compared with the objective of finding motifs that might be employed as diagnostic sequences for the identification of other candidate V-PPase homologs.

Four regions of particularly high sequence conservation among PVP, AVP and RVP were identified (Fig. 5). The first, corresponding to positions 185–231 of PVP, spanned cytosolic loop III and encompassed both the consensus $DX_7KXE$ motif [2] and the antibody $PAB_{TK}$-reactive sequence [16]. The second, corresponding to positions 439–456 of PVP, was encompassed by cytosolic loop VI. The third and fourth, corresponding to positions 644–655 and 665–677 of PVP, the latter of which coincided with the antibody $PAB_{HK}$-reactive sequence, were located in the C-terminal tail.

A systematic database search using these query sequences yielded matches in a range of bacteria and eukaryotes. Within the bacteria, all of the search sequences identified cognates not only in the genome of *T. maritima* (Section 1), but also in the green sulfur bacterium *Chlorobium tepidum* and the α-proteobacterium *Caulobacter crescentus* (Fig. 5). Notable, however, was the absence of V-PPase-like sequences from the recently completed sequenced genomes of several other α-proteobacteria, including *Rickettsia prowazekki*, *Bacillus subtilis* and the deeply divergent bacterium, *Aquifex aeolicus*, as well as those of the archaea *Archaeoglobus fulgidus* [30], *Methanobacterium thermoautotrophicum* [31], *Methanococcus jannaschii* [32] and *Pyrococcus horikoshii* [33]. Among non-plant eukaryotes, a match was found in the apicoplexan protist *Plasmodium falciparum*.

## 4. Discussion

Sequence, computer-assisted topological and functional analyses demonstrate that *PVP*, a gene from the hyperthermophilic archaeon *P. aerophilum*, encodes a sequence-divergent V-PPase ortholog. By exploiting the facility with which PVP is expressed in a functional state in *S. cerevisiae*, under conditions identical to those used for the heterologous expression of AVP, it is shown that the membrane-associated archaeal protein is thermostable and sufficient for fluoride-insensitive PPi hydrolysis at temperatures optimal for the growth of *P. aerophilum*. As a result of defining the basic requirements for PVP-mediated PPi hydrolysis and establishing a strict association between *PVP* expression and the presence of a membrane-associated, $M_r$ 64 000, antibody $PAB_{TK}$- (and $PAB_{HK}$) reactive polypeptide in pYES2-PVP-transformed *S. cerevisiae*,

it has also been determined that the membrane fraction from *P. aerophilum* contains considerable amounts of PVP protein and activity. Since the V-PPase of plant vacuolar membranes represents 1–10% of membrane protein and has an activity of 0.1–1.0 μmol PPi/mg/min [3], values commensurate with those obtained with the endogenous archaeal ortholog, PVP is evidently a major membrane component in *P. aerophilum*.

The characterization of PVP is of considerable strategic and conceptual value. First, because of the divergence of the overall sequence of PVP from that of AVP and other plant V-PPases, it constitutes a reference against which candidate residues or motifs necessary for core catalysis or type-specific functions may be delineated. In the context of core catalysis, examples include but are not limited to the following. (i) The fundamental correspondence of the putative topology of PVP to that of AVP and other plant V-PPases [4], suggesting a basic uniformity of secondary structure despite the marked differences in primary structure. (ii) Retention of the PPase 'catalytic' motif, $DX_7KXE$ [2], in cytosolic loop III, the loop exhibiting greatest sequence conservation between PVP and other V-PPases, implying that this loop constitutes, or at least contributes, to the substrate-binding and/or hydrolysis domain. (iii) Conservation of the motif T(D/E)YYTS at the span-loop interface of cytosolic loop V of PVP, whose equivalent in AVP encompasses the acidic residue ($Glu^{427}$) inferred to play a role in DCCD-insensitive $H^+$-translocation [18], indicating a coupling role for this motif and, as is the case for AVP E427D mutants [18], an enhanced capacity for $H^+$-translocation.

In the context of type-specific functions, the insensitivity of PVP to $K^+$ and other monovalent cations, its decreased sensitivity to inhibition by AMDP and complete insensitivity to maleimides versus AVP and other plant V-PPases [4] offers the potential of delimiting segments of AVP and other plant V-PPases that might be involved in these interactions. PVP's status as a natural AVP $Cys^{634}$-less mutant, active in catalysis but resistant to NEM, is one example. Another is the sensitivity of PVP to inhibition by DCCD despite its possession of only one of the two acidic residues whose conservative substitution in AVP is associated with a diminution of DCCD inhibitability and the near complete abolition of catalytic activity [18]. Apparently, the equivalent of AVP $Glu^{305}$ is dispensable in some but not all V-PPases.

Second, as exemplified by the results shown in Fig. 5, the sequence data from PVP, in combination with those from RVP and plant V-PPases, provide meaningful criteria for the identification of other sequence-divergent V-PPases. The sequences of RVP and, for example, AVP, alone, were not sufficient for this purpose because of the high level of sequence conservation among plant V-PPases (Section 1), the lack of data confirming bona fide PPase activity for RVP [8] and the fact that alignments between only two divergent sequences do not permit systematic conservations to be distinguished from coincidental ones. A particularly important insight gained from these analyses is an appreciation that antibodies $PAB_{TK}$ and $PAB_{HK}$, because they were raised against two of the most sequence-conserved, immunogenic peptide 15-mers identified to date in V-PPases, fulfill the requirements of universal probes for this category of pump.

Third, its pronounced thermostability, capacity for high levels of expression in *S. cerevisiae* and sufficiency for reconstitution of core catalysis make PVP a strong candidate for crystallographic studies of this category of pump, in particular, and a good model for analyses of the structural factors that confer thermostability on integral membrane proteins, in general.

The existence of a V-PPase ortholog in *P. aerophilum* may necessitate revision of our understanding of the evolutionary origins of this category of pump. While V-PPases were formerly thought to occur only in plants, their algal antecedents and *R. rubrum* [3,4], *P. aerophilum*'s possession of a V-PPase and the identification of cognate sequences in the genomes of *C. tepidum*, *C. crescentus*, *P. falciparum* and *T. maritima* (Fig. 5), together with reports of V-PPase-like activities in *T. cruzi* [11] and *S. gentianae* [12], suggest that these pumps are widely distributed and represented in each of the three domains of life [34]. The number of known V-PPase sequences is still too small to speculate meaningfully on their line of descent but in the case of the archaeal, bacterial and eukaryal prototypes, PVP, RVP and AVP, the finding that they have equally divergent (58% similar) sequences is at least consistent with the notion of radiation from a common ancient ancestral gene. Alternate schemes invoking an isolated lateral gene transfer event in *P. aerophilum* cannot yet be discounted, pending the analyses of more crenarchaeotal genomes, but it is unlikely that such transfers are the general case given the presence of V-PPase-like activities or coding sequences in bacteria and in euryarchaeota other than the four whose genomes have been sequenced in entirely (Drozdowicz, Y.M. and Rea, P.A., unpublished). Thus, V-PPases and PPi-energized $H^+$-translocation, rather than being evolutionary backwater phenomena, relics of a mode of energy transduction of limited phylogenetic impact, appear to be much more deeply rooted in the tree of life and prevalent than appreciated hitherto.

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