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Roles of basic residues and salt-bridge interaction in a vacuolar H^+-pumping pyrophosphatase (AVP1) from *Arabidopsis thaliana*

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Abstract

To investigate the possible role of basic residues in H^+ translocation through vacuolar-type H^+-pumping pyrophosphatases (V-PPases), conserved arginine and lysine residues predicted to reside within or close to transmembrane domains of an *Arabidopsis thaliana* V-PPase (AVP1) were subjected to site-directed mutagenesis. One of these mutants (K461A) exhibited a "decoupled" phenotype in which proton-pumping but not hydrolysis was inhibited. Similar results were reported previously for an E427Q mutant, resulting in the proposal that E427 might be involved in proton translocation. However, the double mutant E427K/K461E has a wild type phenotype, suggesting that E427 and K461 form a stabilising salt bridge, but that neither residue plays a critical role in proton translocation.

Keywords: *Arabidopsis thaliana*; AVP1; Proton pump; V-PPase; Salt bridge; Site-directed mutagenesis

1. Introduction

Plant vacuolar H^+-pumping inorganic pyrophosphatases (V-PPases) play central roles both in vacuolar acidification and probably in the control of cytosolic PPi concentration [1]. These endomembrane enzymes are ubiquitously distributed among higher plants, and are additionally present in the photosynthetic bacterium *Rhodospirillum rubrum* [2], in some Archea [3] and in parasitic protists where they also contribute to a PMF across the plasma membrane [4]. In *Arabidopsis thaliana*, three *AVP* genes encode members of the V-PPase family, which is highly conserved in plant species [5,6]. AVP1 is an extremely hydrophobic protein of 770 residues (M_r=80,800). Heterologous expression of AVP1 in the yeast *Saccharomyces cerevisiae* has demonstrated that this polypeptide is sufficient for both H^+ pumping and PPi hydrolysis [7]. Early models favoured the presence of 13 transmembrane domains (TMD) for AVP1 [8], although more refined models proposed for vacuolar PPases suggest either 15 [1,9] or 16 TMD [10].

Sequence comparisons among V-PPases have provided valuable information concerning likely critical domains and residues. To date over 100 sequences from bacteria, archea and eukaryotes are available [11]. Sequence alignment has revealed two sub-families of V-PPase, one of which contains exclusively K^+-dependent enzymes, the other of which contains K^+-independent enzymes [12,13]. Intriguingly, members of the K^+-independent sub-family all contain a Lys residue at a position equivalent to residue 541 of AVP1. Furthermore, substitution of a neutral residue by Lys in this position in the K^+-dependent V-PPase from *Carboxydothermus hydrogenoformans* confers K^+-independency [12]. However, other residues clearly contribute to the K^+ binding site, including (from sequence alignments) G544 and various Cys residues towards the N terminus [13].

The sequence between TMDs V and VI is extensively conserved and, by analogy with soluble PPases, is likely to contain the substrate binding site [14]: the sequence 257DX(A/G)(A/G)DL(S/V)GKXE267 is conserved in all V-PPases sequenced to date. Alanine substitutions of the first Asp residue in the sequence or the Lys or Glu residues result in a non-functional

Abbreviations: AO, acridine orange; DCCD, dicyclohexylcarbodiimide; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PVDF, polyvinylidiene difluoride; TMD, transmembrane domain

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enzyme [15]. In the latter two cases, the enzyme fails to exhibit substrate protection from tryptic digestion that occurs in the wild type enzyme. Furthermore, mutagenesis of H^+-PPase from *Rhodospirillum rubrum* led to the conclusion that conserved Glu residues at positions 197 and 202 [16] and an Asp residue at position 204 [17] (corresponding to E267, E272 and D274 in AVP1) might contribute to substrate binding by controlling Mg^{2+} binding. Two further regions, both acidic and extensively conserved, are present in putative extramembranous loops: 283DXVGDNVGD291 (also between TMDs V and VI) and 726GDT(I/V)GDPXKDTXGP739 (C-terminal to TMD XV). These have been subjected to site-directed mutagenesis and in each case neutralisation of the Asp residues also results in loss of hydrolytic and transport activities [15,18]. These findings, together with more mild effects on substrate-protectable trypsin susceptibility, suggest further involvement in substrate binding.

By contrast, far less is known of intramembrane residues that might be involved in ion translocation. Zhen et al. [9] mutated eight acidic residues predicted to be in or near TMDs. In two of the three mutants in which activity was modified (E305 and D504), H^+ pumping was abolished and hydrolytic activity much reduced. The mutant E427Q was markedly more impaired with respect to transport than to hydrolysis, thereby leading to the suggestion that E427 is involved in coupling hydrolysis to H^+ translocation.

The involvement of intramembrane basic residues in H^+ translocation and hydrolysis has recently been addressed in H^+-PPase from *R. rubrum*. Thus, R176 (R246 in AVP1) has been indicated as a putative residue involved in coupling and substrate binding [18]. Furthermore, a salt bridge between R176 and possibly E584 (E645 in AVP1), located at the membrane–cytosol interface of TMD V and XIV respectively, has been proposed [18]. In the vacuolar PPase from *Vigna*, the replacement of R242 (corresponding to R246 in *A. thaliana*) to A leads to a marked decline in enzymatic and proton translocation activities [19]. Since this residue is located at the end of a highly conserved TMD, these authors speculated that R242 might be directly or indirectly involved in cation binding and salt bridge formation [19].

In the present investigation, we have extended previous work on the roles of intramembrane charged residues in the coupling of hydrolysis to proton pumping. Three basic residues in AVP1 were selected for study (Fig. 1): R192 is within or close to TMD IV, R246 in TMD V and K461 in the region of TMD X. In each case, the residue was mutated either to another basic residue or charge-neutralised to Ala and each mutant then assayed for hydrolytic and H^+-pumping capacities. The results of these studies and of those on a double mutant E427K/K461E suggest that caution should be exercised in ascribing catalytic function to specific residues, and require revision of the hypothesis that E427 is critical for H^+ translocation: rather it appears that E427 and K461 form a salt bridge that is involved in enzyme stabilisation.

2. Materials and methods

2.1. Microorganisms and heterologous expression in yeast

S. cerevisiae, haploid strain BJ5459 (*MATa, ura3-52, trp1, lys2-801, leu2Δ1, his3-Δ200, pep4::HIS3, prbΔ1.6R, can1, GAL*), was transformed with the yeast-*E. coli* shuttle vector pYES2 containing the entire open reading frame of AVP1 inserted between the *GAL1* promoter and *CYC1* termination sequences (pYES2-AVP1) as described by Kim et al. [20].

2.2. Site-directed mutagenesis

Site-directed mutagenesis was performed using a Quik Change Site-Directed Mutagenesis Kit from Stratagene. The sequences of the oligonucleotide primers used for mutagenesis in this study are shown in Table 1. Mutagenesis was confirmed by sequencing the mutated region prior to yeast transformation.

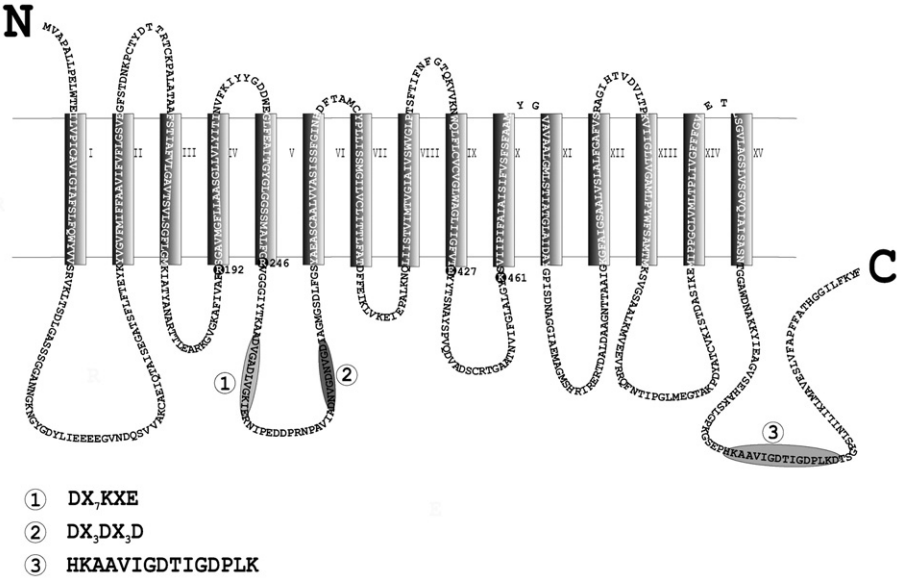

Fig. 1. Putative membrane topology of AVP1, drawn according to Drozdowicz and Rea [30]. Mutated residues are indicated in black circles. (1) Putative catalytic motif DX_7KXE [14]; (2) Acidic motif DX_3DX_3D [15]; (3) Conserved sequence recognized by V-PPase-specific antiserum used in the present study (PAB_{HK} [7]).

Table 1
Primers used for site-directed mutagenesis of AVP1, heterologously expressed in yeast

Mutation	Primer sequence (5′ to 3′)
R192K	CATTGTTGCATTC**AAG**TCTGGTGCTGTG
R192A	CATTGTTGCATTC**GCG**TCTGGTGCTGTG
R246K	GCTCTCTTTGGC**AAG**GTTGGTGGTGGG
R246A	GCTCTCTTTGGC**GCT**GTTGGTGGTGGG
K461R	GCTCTTGGTTAC**AGA**TCCGTCATTATTC
K461A	GCTCTTGGTTAC**GCT**TCCGTCATTATTC
E427K/K461E	GGTTTCGTCACT**AAG**TACTACACTAG
	GCTCTTGGTTAC**GAG**TCCGTCATTATTC

Substitutions involving no change in charge are in italics. The nucleotides changed for the substitution are in boldface.

2.3. Preparation of vacuolar membrane vesicles

Yeast vacuolar membrane-enriched vesicles were prepared as described by Kim et al. [20]. Briefly, yeast cell culture obtained from 2 l of AHC medium supplemented with galactose were grown for 2 days and collected by centrifugation. After cell wall digestion, the cells were homogenized in 5 mM Tris–MES (pH 7.6), 10% (w/v) glycerol, 5 mM EGTA and 2 mg/ml BSA containing 1 mM PMSF and 1 μg/ml leupeptin. Microsomes were obtained after centrifugation of the supernatant at 4000×*g* for 10 min and 100,000×*g* for 40 min. The microsome fraction was then centrifuged at 100,000×*g* for 2 h on a discontinuous sucrose density gradient consisting of 10% (w/v) and 28% (w/v) sucrose in 5 mM Tris–MES (pH 7.6), 1.1 M glycerol, 2 mM DTE, 1 mM EGTA and 2 mg/ml BSA. Partially purified vacuolar membranes were collected at the 10–28% interface and ultracentrifuged at 100,000×*g* min for 1 h. The final pellet was resuspended in 5 mM BTP–MES (pH 7.6), 1.1 M glycerol and 2 mg/ml BSA and immediately stored at −80 °C.

2.4. Measurement of PPase activity and protein determination

Pyrophosphatase activity was evaluated as Pi release from PPi at 37 °C in 30 mM Tris–Mes (pH 8.0), 100 mM KCl, 1.3 mM $MgSO_4$, 0.1 mM EGTA, 5 μg gramicidin-D, 1 mM KF and 20 μg of membrane protein in a final volume of 300 μl. The reactions were initiated by the addition of 0.3 mM PPi and blocked by the addition of the Ames reagent [21]. Hydrolysed PPi was calculated as half of the amount of Pi released by PPase activity.

PPi-dependent proton translocation was evaluated as acridine orange (AO) fluorescence quenching [6] using a Perkin-Elmer LS5 fluorimeter. The medium was 30 mM Tris–Mes (pH 8.0), 0.4 M glycerol, 100 mM KCl, 1.3 mM $MgSO_4$, 0.1 mM EGTA, 5 μM AO and 100 μg membrane protein in 500 μl final volume. The reactions were performed at 25 °C and started by the addition of 1 mM PPi.

Protein was estimated using the Bradford method [22], using bovine serum albumin as a standard.

2.5. Western blot analyses

Membrane samples (5 μg) were electrophoresed on 5–15% (w/v) SDS-PAGE and then electrotransferred to PVDF membrane in a semi-dry blotting apparatus. The filters were then processed with PPase polyclonal antibodies raised in rabbit against a synthetic oligopeptide corresponding to the conserved motif HKAAVIGDTIGDPLK ([7]: see Fig. 1). Immunoreactive bands were detected by chemiluminescence resulting from the horseradish peroxidase conjugate-catalysed breakdown of luminol (ECL System, Amersham). Densitometric analysis of the Western blot was performed by the Quantity One program (Bio-Rad).

3. Results

To determine the possible role of basic residues in V-PPase-mediated H^+ translocation, we used a 15 TMD topological model [9] to identify three basic residues located at the trans-membrane helix–cytosol interface, namely R192, R246 and K461. Each was mutated either to the corresponding positively charged residue (R to K, or K to R) or to a neutral residue (A). Vacuolar membrane-enriched vesicles obtained from yeast expressing wild type or mutant proteins were used to evaluate the PPase enzymatic activity either as proton pumping (AO fluorescence quenching) or as PPi hydrolysis. As a control, we established that very similar Bafilomycin A_1-inhibitable V-ATPase hydrolytic and ΔpH generation activity native to these membranes was present in vacuolar-enriched vesicles prepared from yeast expressing the mutated and non-mutated yeast forms of V-PPase (not shown). These findings indicate that the vesicles were uniformly viable and were not leaky to protons. We also confirmed with Western blotting that all mutated proteins were expressed in the vacuolar yeast membranes (Fig. 2). In all cases, expression levels appeared similar, with the exception of the R246A mutant, for which a significantly lower level of expression was recorded. Densitometric analysis of Western blots suggested approximately 45% decrease in R246A mutant protein content with respect to either wt or R246K mutant. This decrease might be related to mistargeting or to more rapid turnover. No immunoreactive band was present in yeast transformed with the empty vector pYES2 (Fig. 2, ev).

Fig. 3 shows that mutations in residue R192 had little, if any, effect on the rate and extent of H^+ pumping. A corresponding lack of effect on hydrolytic activity (Table 2) suggests that R192 is not a critical residue in V-PPase function, despite the fact that sequence alignments (not shown) indicate conservation of a basic (R or K) residue in this position in nearly all V-PPases sequenced so far.

By contrast, mutation of residue R246 had a profound effect on H^+ pumping (Fig. 3). The initial rate of H^+ pumping was decreased to between 2% and 3% that of the wild type enzyme, independently of whether mutation involved neutralisation of charge or not. There was a correspondingly dramatic decline in hydrolytic activity to around 14% of control in the case where the positive charge was neutralised as R246A, while retention of positive charge in the R246K mutant led to less, albeit still highly significant, inhibition by a factor of two (Table 2). Even when corrected for the lower expression level, the proton pumping rate of the R246A mutant was still only 4% of wt and hydrolytic activity was 26% of control. These results might suggest an essential role for R246 in V-PPase function, and the point is returned to in Discussion.

The role of residue K461 presents, at first sight, an interesting variation on those of R192 and R246. Fig. 3 and Table 2 show

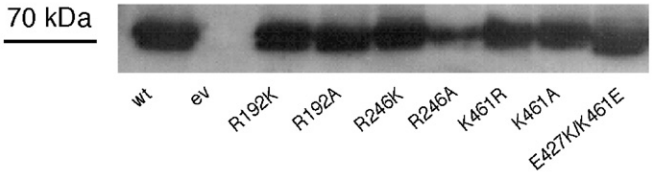

Fig. 2. Western blot of vacuolar membrane-enriched vesicles (5 μg each lane) prepared from pYES2-AVP1 transformed *S. cerevisiae* BJ5459 cells expressing either wild type (wt), empty vector (ev) or mutated V-PPase protein.

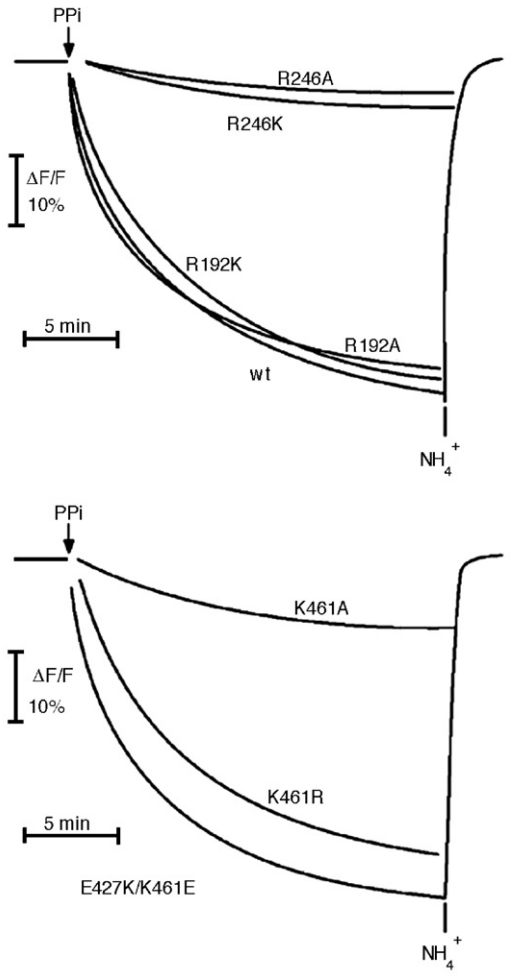

Fig. 3. Proton-pumping activity of AVP1 (wt) and site-directed mutants, evaluated as AO fluorescence quenching. Arrow indicates addition of 1 mM PPi. Dissipation of the PMF, indicated by relaxation of fluorescence quenching, was initiated by addition of 10 mM NH^+_4.

that for the mutant K461R in which charge is retained, neither H^+ pumping nor hydrolytic activity is markedly affected, suggesting a non-essential role for this residue. By contrast, neutralisation of charge in the K461A mutant yields a dramatic effect on H^+ pumping, with a reduction in initial rate to only about 5% that of the wild type control. Furthermore, as shown in Table 2, hydrolytic activity remains at the control level: this particular mutation results in a protein in which PPi hydrolysis has effectively been uncoupled from H^+ translocation. This raises the possibility that the positive charge on K461 is intimately involved in transmembrane H^+ transport, with the ε amino group undergoing reversible protonation as part of the transport process—a function that could then be conserved in the K461R mutant.

However, inspection of Fig. 1 reveals that K461 is potentially close to a second residue, E427, which, when mutated to Q has been revealed to yield enzyme with similar "uncoupled" properties [9]: the two residues are associated with two potentially adjacent TMDs that are separated only by a short hydrophilic loop. Is it possible that charge–charge interactions in the form of a salt bridge between the two residues accounts for the uncoupled properties of the mutants, rather than a specific role of either residue in H^+ translocation *per se*?

To test this idea, we constructed a mutant in which the residues were swapped with respect to their positions in wild type AVP1, and hence the charges interchanged. Fig. 3 shows that for this double E427K/K461E double mutant, H^+ pumping was restored essentially to wild type levels, and hydrolytic activity possibly slightly enhanced. These results suggest that neither E427 nor K461 plays an essential role in the H^+ translocation pathway: rather that interactions between the two residues hold the enzyme in a conformation favourable for H^+ translocation, but that disruption of the interaction has no effect on hydrolysis. Furthermore, the high proton pumping activity shown by the double mutant even in the presence of high KCl concentration (100 mM) might be explained by a very limited access of such a salt to these residues. Despite their predicted positions at the membrane/cytosol interface, both E427 and K461 are flanked by non-polar or uncharged polar residues (see Fig. 1) and hence may be located within a hydrophobic pocket located near the lipid bilayer and cytosol interface.

4. Discussion

Mutation of three basic residues within or close to TMDs of the *Arabidopsis* V-PPase AVP1 has revealed dramatically different potential roles. In the case of R192, even neutralisation of the positive charge failed to affect either the hydrolytic or the proton pumping activity of the enzyme and the residue is clearly not essential for activity. By contrast, both R246K and R246A mutants exhibit severely curtailed hydrolysis and transport (Table 2 and Fig 3). These results are partially in agreement with those from H^+-PPase of *R. rubrum*, where mutation of R176 (corresponding to R246 in AVP1) to Ala completely abolishes both hydrolytic and transport activities, while the R176K mutant exhibits selective impairment of proton translocation [18]. Alignment of 41 V-PPase sequences from plants, protists and Archea demonstrates that R246 is conserved in all but two (where it is substituted by Q). [Interestingly, one the V-PPases in

Table 2
PPase activity in wild type and mutated AVP1, heterologously expressed in yeast, evaluated either as proton pumping (AO fluorescence quenching) or as PPi hydrolysis (Pi released)

	$\Delta F\%$ min^{-1} mg^{-1} protein	nmol PPi hydrolysed min^{-1} mg^{-1} protein	$\Delta F\%$ $nmol^{-1}$ Pi hydrolysed
Wild type	585	125.1±6.0	4.7
Empty vector	N.D.	19.0±1.0	N.A.
R192K	507	102.9±1.1	4.9
R192A	588	121.4±6.9	4.8
R246K	17	57.6±6.8	0.3
R246A	13	18.0±0.9	0.7
	24*	(32.7±1.6)*	
K461R	425	98.5±11.4	4.3
K461A	27	127.3±4.5	0.2
E427K/K461E	570	149.5±6.3	3.8

N.D., not detectable; N.A., not applicable. *, values corrected for the expression level of R246A mutant, estimated to be approximately 55% of wt.

which this residue is not conserved is the *Arabidopsis* isoform AVP2, ref. 23.] Although this residue is predicted to lie just within TMD V, it is also close to the start of a very highly conserved sequence of over 60 residues that subsumes the PPi binding site [6]. Mutations of other residues within this sequence (D257, K265) yield non-functional enzyme [15]. Thus, it is possible that R246 is instrumental in providing a transmembrane pathway for H^+ or, as recently proposed for both *R. rubrum* [18] and *V. radiata* [19] PPases, it could be involved in salt bridge formation. Nevertheless, it is equally possible that this Arg residue contributes to the catalytic site. The latter notion is supported by the dramatic inhibitory effect of the R246A mutation on hydrolytic activity of AVP1.

Residue K461 presents an interesting case. While catalytic activity is tolerant of a charge-conserving substitution, neutralisation of the charge is selective in inhibiting H^+ transport and not PPi hydrolysis. At first sight, K461 might therefore be considered as a good candidate residue for involvement in H^+ translocation. Indeed similar results (i.e. inhibition of transport with little effect on hydrolysis) were obtained for the E427Q mutant and this led the authors to speculate that E427 might be involved in coupling H^+ translocation with PPi hydrolysis [9].

Sequence alignments show that, although K461 is conserved in a number of V-PPases, the equivalent residue in AVP2 is negatively charged (E475: [23]). Moreover, in AVP2, E427 is apparently substituted by a positively charged residue (K441). Similarly, in the *Plasmodium* V-PPase PfVP2, the equivalent residues are respectively E724 and R690 [4], again showing evidence for interchange of charge. These observations, together with hydropathy analysis indicating that the two residues might be associated with closely juxtaposed helices, led us to attempt restoration of H^+ pumping activity by construction of an AVP1 mutant in which the charges at the two positions were interchanged. In this we were successful: the E427K/K461E mutant has hydrolytic and transport characteristics similar to wild type AVP1.

It therefore seems unlikely that either E427 or K461 plays an essential or direct role in H^+ translocation and much more likely that an interaction between them in the form of a salt bridge is responsible for maintaining the enzyme in a conformation in which H^+ translocation can be facilitated. There are numerous cases of membrane proteins in which such salt bridge formation has been proposed on the basis of site-directed mutagenesis that involves charge swapping or joint neutralisation of positive and negative charges. Examples include the vesicular monoamine transporter 2 [24], the α_{1b} adrenergic receptor [25] and the *E. coli* melibiose carrier [26]. In some examples, the double mutants have been shown to be completely functional, namely, mutant D237K/K358D in lactose permease of *E. coli* [27] and mutant R347D/D924R of the cystic fibrosis transmembrane conductance regulator [28] that still retain enzymatic activities similar to the corresponding wild type proteins. Similarly, yeast plasma membrane proton pumping ATPase – a 100-kDa 10 TMD polypeptide – shows complete activity when the polarity of R695 and D730 is reversed, but not when the charge on either is changed individually, suggesting that those residues are involved in a salt bridge that connects TMDs 5 and 6 [29].

It is important to note however, that our proposition for a salt bridge between residues E427 and K461 in AVP1 (and by extension K441 and E475 in AVP2 and R690 and E724 in PfVP2) is not a universally conserved feature of V-PPases. Alignment of over 40 sequences shows a number of instances in which the acidic residue at the position equivalent to E427 is conserved, while that at the position equivalent to K461 is also acidic. Clearly, a number of factors contribute to protein folding and stabilisation in the vicinity of these residues, and a full picture will not emerge until the three-dimensional structure is solved.

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