EVIDENCE FOR A CONSERVED 95–120 kDa SUBUNIT ASSOCIATED WITH AND ESSENTIAL FOR ACTIVITY OF V-ATPases

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Summary

Vacuoles purified from Saccharomyces cerevisiae bearing the vph1-1 mutation had no detectable bafilomycin-sensitive ATPase activity or ATP-dependent proton pumping. Furthermore, the vacuolar H+-ATPase (V-ATPase) nucleotide binding subunits were no longer associated with vacuolar membranes yet were present at wild-type levels in yeast whole-cell extracts. The VPHI gene was cloned by screening a λ gt11 expression library with antibodies directed against a 95 kDa vacuolar integral membrane protein and independently cloned by complementation of the vph1-1 mutation. Deletion disruption of the VPH1 gene revealed that the VPH1 gene is required for vacuolar H+-ATPase assembly and vacuolar acidification but is not essential for cell viability or for targeting and maturation of vacuolar proteases. VPH1 encodes a predicted polypeptide of 840 amino acid residues (95.6 kDa) with putative membrane-spanning regions. Cell fractionation and immunodetection demonstrate that Vph1p is a vacuolar integral membrane protein that co-purifies with V-ATPase activity. Vph1p has 42 % identity to the 116 kDa polypeptide of the rat clathrin-coated vesicles/synaptic vesicle proton pump, 42% identity to the TJ6 mouse immune suppressor factor, 42% identity to the Caenorhabditis elegans proton pump homologue and 54% identity to the predicted polypeptide encoded by the yeast gene STV1 (Similar To YPH1, identified as an open reading frame next to the BUB2 gene).

Introduction

The first accounts of V-ATPase structure did not report any associated subunits larger than the 70 kDa nucleotide binding subunit. Susceptibility of the 95–120 kDa subunit to proteolysis and to aggregation may be in part attributed to its absence in these early reports. More recently, the 95–120 kDa subunit has been shown to co-purify with V-ATPases from several sources and to be essential to V-ATPase activity in both biochemical and genetic experiments. Here we review the recent evidence that a conserved 95–120 kDa integral membrane polypeptide is associated with V-ATPases from various organelles and is essential to the assembly and activity of the enzyme.

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Key words: V-ATPase, H+-ATPase, vacuole, yeast, Saccharomyces cerevisiae.

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Possible explanations for the absence of the 95–120 kDa subunit from purified V-ATPases from specific sources

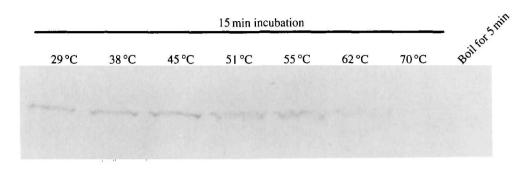
The first reports of V-ATPase structure (Uchida et al. 1985; Marin et al. 1985; Manolson et al. 1985) were generally one-step purifications on glycerol gradients or Sepharose columns resulting in low purifications and complex polypeptide mixtures. The subunits reported to be associated with these V-ATPases were conservatively limited to the polypeptides that could be specifically labeled with radioactive probes such as the 70 and 60 kDa nucleotide binding subunits and the 16 kDa N,N'-dicyclohexylcarbodiimide binding subunit. If one re-examines some of the protein gels from these first reports, a subunit of approximately $100 \times 10^3 M_{\rm r}$ can indeed be seen in the lanes containing V-ATPase activity (Manolson et al. 1985, Fig. 8). Nevertheless, there are more recent reports on bovine renal tubules (Gluck and Caldwell, 1987), rat Golgi apparatus (Moriyama and Nelson, 1989a) and rat liver lysosomes (Moriyama and Nelson, 1989b) in which purified V-ATPases from these sources did not contain a subunit migrating between 95 and $120 \times 10^3 M_r$. For rat liver lysosome and Golgi apparatus V-ATPases, the absence of a $95-120\times10^3 M_r$ polypeptide may reflect a genuine difference in the composition of V-ATPases from various sources. Gillespie et al. (1991) have since presented immunological evidence of a $115\times10^3 M_r$ subunit associated with bovine renal tubule V-ATPase, suggesting that the absence of this subunit, as reported by Gluck and Caldwell (1987), may have resulted from the difficulty of visualizing the $95-120\times10^3 M_r$ subunit by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Susceptibility of the 95-120 kDa subunit to proteolysis during V-ATPase purification has been reported by Gillespie et al. (1991) and Kane et al. (1992). Adachi et al. (1990a) have shown that the $100 \times 10^3 M_r$ subunit from purified bovine coated vesicle V-ATPase is quickly degraded to an $80 \times 10^3 M_{\rm r}$ and a $21 \times 10^3 M_{\rm r}$ species by low concentrations of trypsin. To avoid the proteolysis problem when purifying the Saccharomyces cerevisiae vacuolar H⁺-ATPase, we have used a yeast strain (BJ926, $MAT\alpha/MATa$, trpl-1/+ +/hisI, prc1-126/prc1-126 pep4-3/pep4-3, prb1-1122/prb1-1122 can1/can1 gal2/gal2) in which the structural genes encoding the predominant yeast vacuolar hydrolases (protease A, protease B and carboxypeptidase Y) have been mutated. Furthermore, to reduce other $1 \text{ mol } 1^{-1}$ EDTA. cytosolic protease activities. we included phenylmethylsulfonyl fluoride and $1 \mu g \, ml^{-1}$ pepstatin A during the V-ATPase purification procedure (Manolson et al. 1992).

A second potential problem for visualizing the $95-120\times10^3\,M_{\rm r}$ V-ATPase subunit on SDS-PAGE is its tendency to aggregate when heated in protein-denaturing sample buffer (Gillespie *et al.* 1991; Manolson *et al.* 1992). Denaturing whole-cell extracts in the presence of 1 % SDS and $0.6\,{\rm mmol}\,1^{-1}\,\beta$ -mercaptoethanol at temperatures over 62 °C, or at 62 °C for longer than 15 min, resulted in the failure to detect any signal on an immunoblot from the yeast 95 kDa V-ATPase subunit, Vph1p (Fig. 1). Although 45 °C for 15 min and 62 °C for 2.5 min appear to result in equal signals (Fig. 1), the latter treatment was found to be empirically more reproducible.

Evidence that a 95-120 kDa subunit is associated with V-ATPases

Purifications of V-ATPases that have resulted in an associated $95-120\times10^3 M_r$ subunit are listed in Table 1. Monoclonal (Kane *et al.* 1992) and polyclonal (Manolson *et al.* 1992) antibodies to a yeast $95-100\times10^3 M_r$ polypeptide show co-purification of the



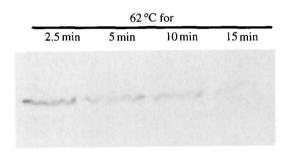


Fig. 1. The 95 kDa yeast V-ATPase subunit, Vph1p, requires brief low-temperature incubations for solubilization in SDS-PAGE denaturation buffer. 150 μ g of whole-cell extracts (prepared as described in Manolson *et al.* 1992) were denatured for SDS-PAGE in 63 mmol1⁻¹ Tris-HCl pH 6.8, 1 % SDS, 0.6 mmol1⁻¹ β -mercaptoethanol and 5 % glycerol for 15 min at 29, 38, 45, 51, 55, 62 or 70 °C (top panel), boiling for 5 min (top panel, right-hand lane), or at 62 °C for 2.5, 5, 10 or 15 min (bottom panel). Samples were subjected to SDS-PAGE, transferred to nitrocellulose and then subjected to immunoblot detection using a 1:350 dilution of affinity-purified anti-Vph1p.

Table 1. V-ATPases with associated 95-120 kDa subunits

Organelle	Source	Molecular mass×10 ⁻³	Reference
Clathrin-coated vesicles	Bovine brain	100–116	Xie and Stone (1986); Arai <i>et al.</i> (1987); Perin <i>et al.</i> (1991)
Chromaffin granule	Bovine adrenal medulla	140 115	Percy et al. (1985); Moriyama and Nelson (1987)
Chromaffin granule	Human phaeochromocytoma	115	Gillespie et al. (1991)
Renal tubule	Bovine renal medulla	115	Gillespie et al. (1991)
Vacuole	Red beet storage tissue	100	Parry et al. (1989)
Vacuole	Yeast	95–100	Kane et al. (1992); Manolson et al. (1992)

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8	Consen	PAVAFCDAGHGEPIAFI-ALF LVLAEKKAMK-RDEIFDA -FTGRYIILLAG-F8-YTGL-540				

Fig. 2

antigen with V-ATPase activity whereas immunofluorescence microscopy (Kane *et al.* 1992) and immunogold electron microscopy (Manolson *et al.* 1992) have demonstrated the specificity of the antigen for the yeast vacuolar membrane. The V-ATPase nucleotide binding subunits appear to block epitope sites *in vivo* on the yeast $95-100\times10^3 M_r$ polypeptide (Kane *et al.* 1992), further supporting the association of this polypeptide with the yeast vacuolar H⁺-ATPase.

Evidence that a 95–120 kDa subunit is required for assembly and activity of V-ATPases

Specific removal of the 116 and $38 \times 10^3 M_r$ subunits from the purified clathrin-coated V-ATPase did not affect Ca²⁺-activated ATP hydrolysis yet eliminated Mg²⁺-supported ATP hydrolysis and proton pumping activity (Xie and Stone, 1988). Mutations in the structural gene encoding the yeast 95 kDa subunit, *VPH1*, result in a pH of 6.9 in the vacuolar lumen instead of the wild-type pH of 6.2 (Preston *et al.* 1989). Vacuoles purified from strains bearing the *vph1-1* mutation had no measurable bafilomycin-sensitive ATP hydrolysis or proton-pumping activity (A. K. Bachhawat, M. F. Manolson, D. G. Murdock, D. Garmen and E. W. Jones, in preparation) and had barely detectable levels of the 60 and 69 kDa peripherally bound nucleotide-binding V-ATPase subunits (Manolson *et al.* 1992). In contrast, when either of the structural genes encoding the 60 and 69 kDa V-ATPase (*VAT2*, *TFP1*) is disrupted, the 95 kDa subunit is correctly targeted and stably inserted into the vacuolar membrane as long as the 16 kDa subunit is present (Kane *et al.* 1992). Taken together, these results suggest that the 95 and 16 kDa subunits are epistatic to the 60 and 69 kDa subunits for V-ATPase assembly.

The 95-120 kDa V-ATPase subunit is an integral membrane protein

The $100\times10^3M_{\rm r}$ subunit of the coated vesicle proton pump was labeled by the hydrophobic reagent 3-(trifluoro-methyl)-3-(m-iodophenyl)diazirine, implying that a portion of the polypeptide is buried within the membrane bilayer (Arai et~al.~1988). Failure of chaotropic agents such as alkaline Na₂CO₃ (Kane et~al.~1992; Manolson et~al.~1992) or $0.3~{\rm mol\, l^{-1}}$ KI (Adachi et~al.~1990b) to strip the $95-100\times10^3M_{\rm r}$ subunit from membranes further suggests that this subunit is an integral membrane protein. This biochemical evidence is supported by sequence analysis of the 116 kDa subunit of the clathrin-coated vesicle/synaptic vesicle H⁺-ATPase (Perin et~al.~1991) and the 95 kDa subunit of the yeast vacuolar H⁺-ATPase (Manolson et~al.~1992). In both cases, the carboxyl terminal half of the protein contains at least six regions of sufficient length and hydrophobicity to be considered as membrane-spanning regions.

Fig. 2. Multiple sequence alignment. The entire lengths of the yeast *VPH1* gene product (Vph1p), the yeast *STV1* gene product (Stv1p), the 116 kDa polypeptide of the rat clathrin-coated vesicle H⁺-ATPase (Rat), the mouse TJ6 mouse immune suppressor factor (Mouse) and the *Caenorhabditis elegans* (Cel) proton pump homologue have been aligned using the PILEUP and PRETTY programs (Genetics Computer Group Sequence Analysis Software Package). The alignment and consensus sequence were created using the default settings. Amino acid members of the winning coalition are in upper case and underlined; the consensus sequence is shown directly below the alignment (Consen).

The 95–120 kDa V-ATPase subunit is evolutionarily conserved amongst V-ATPases from a variety of sources

As shown is Table 1, a 95-120 kDa subunit has been found in V-ATPases from a variety of sources. Antibodies directed against the $120 \times 10^3 M_r$ subunit of the chromaffingranule V-ATPase recognized a $115 \times 10^3 M_{\rm r}$ polypeptide in the renal tubule V-ATPase, implying shared epitopes between the two proteins (Gillespie et al. 1991). There are now five sequences that appear to encode this V-ATPase subunit; the 116 kDa polypeptide of the rat clathrin-coated vesicle/synaptic vesicle proton pump (Perin et al. 1991, accession number M58758), the 95 kDa subunit (Vph1p) of the yeast vacuolar H+-ATPase (Manolson et al. 1992, accession number M89778), the predicted polypeptide encoded by the Similar To VPH1 yeast gene STV1 (M. F. Manolson, D. Proteau, R. A. Preston, B. T. Roberts, M. A. Hoyt and E. W. Jones, in preparation), the TJ6 mouse immune suppressor factor (Lee et al. 1990, accession number M31226) and the Caenorhabditis elegans proton pump homologue (Sulston et al. 1992, accession number Z11115). Multiple sequence alignments demonstrate the extensive homology over the entire open reading frames of these five sequences (Fig. 2). Of the five sequences, only the rat 116kDa polypeptide and the yeast 95 kDa subunit (Vph1p) have been directly linked to their respective V-ATPase enzyme. The TJ6 mouse immune suppressor factor and the Caenorhabditis elegans proton pump homologue are thought to be V-ATPase subunits solely on the basis of sequence homology. Although the Similar To YPH1 yeast gene product, Stylp, was initially identified merely on the basis of sequence homology, there is now genetic evidence that it is a functional homolog of Vph1p (M. F. Manolson, D. Proteau, R. A. Preston, B. T. Roberts, M. A. Hoyt and E. W. Jones, in preparation).

Possible function of the 95-120 kDa V-ATPase subunit

Although there is considerable evidence that a 95–120 kDa subunit is associated with a variety of V-ATPase, its specific function is unknown. Data showing that this one subunit contains domains exposed to the cytoplasm (Adachi et al. 1990a) and to the membrane bilayer (Adachi et al. 1990b; Kane et al. 1992; Manolson et al. 1992) have led Perin et al. (1991) to speculate that its function is in coupling ATP hydrolysis (carried out by the cytoplasmic subunits) to proton translocation (carried out by the intramembranous subunits). Yeast contain several membrane-bound organelles that require electrochemical gradients across their membranes (vacuole, clathrin-coated vesicles, peroxisomes and Golgi complex). The presence of at least two functional homologues of the 95–100 kDa V-ATPase subunit in yeast (Vph1p and Stv1p) has led Manolson et al. (1992) to hypothesize that this subunit may be involved in targeting or regulating V-APases for specific endomembrane organelles. In Saccharomyces cerevisiae, the 95 kDa subunit, Vph1p, is essential for the in vivo assembly and activity of the vacuolar H+-ATPase.

This work was supported by a grant from the Medical Research Council of Canada to M.F.M., grants from the National Institutes of Health, GM29713 and DK18090 (E.W.J.),

and in part by a grant from the Pittsburgh Supercomputing Center through the NIH Division of Research Resources cooperative agreement U41 RR04154.

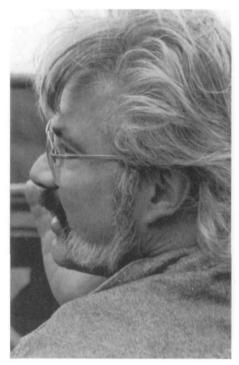
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