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Vacuolar H^+-ATPase: from mammals to yeast and back

N. Nelson[a,*] and D. J. Klionsky[b]

[a]*Department of Biochemistry, Tel Aviv University, Ramat Aviv, 69978 Tel Aviv (Israel)*
[b]*Section of Microbiology, University of California, Davis (California 95616, USA)*

Abstract. Vacuolar H^+-adenosine triphosphatase (V-ATPase) is composed of distinct catalytic (V_1) and membrane (V_0) sectors containing several subunits. The biochemistry of the enzyme was mainly studied in organelles from mammalian cells such as chromaffin granules and clathrin-coated vesicles. Subsequently, mammalian cDNAs and yeast genes encoding subunits of V-ATPase were cloned and sequenced. The sequence information revealed the relation between V- and F-ATPases that evolved from a common ancestor. The isolation of yeast genes encoding subunits of V-ATPase opened an avenue for molecular biology studies of the enzyme. Because V-ATPase is present in every known eukaryotic cell and provides energy for vital transport systems, it was anticipated that disruption of genes encoding V-ATPase subunits would be lethal. Fortunately, yeast cells can survive the absence of V-ATPase by 'drinking' the acidic medium. So far only yeast cells have been shown to be viable without an active V-ATPase. In contrast to yeast, mammalian cells may have more than one gene encoding each of the subunits of the enzyme. Some of these genes encode tissue- and/or organelle-specific subunits. Expression of these specific cDNAs in yeast cells may reveal their unique functions in mammalian cells. Following the route from mammals to yeast and back may prove useful in the study of many other complicated processes.
Key words. Vacuolar H^+-ATPase; biogenesis; assembly; proton pump; bovine; yeast.

Introduction

The eukaryotic cell is defined by a separate nucleus containing most of the genetic material of the cell, semiautonomous organelles containing their own unique DNA and RNA molecules, and a vacuolar system composed of an internal network of membranes. The existence of numerous compartments inside the cell and the variable environments in which eukaryotic cells live requires a constant supply of energy for maintaining the precise concentration of solutes in the various cell compartments. Proton pumps play a major role in providing energy for several secondary uptake processes as well as maintaining the pH homeostasis required for every living cell [1]. There are two mechanistically distinct ATP-dependent proton pumps. One belongs to the family of P-ATPases that operates with a phosphoenzyme intermediate, and the second belongs to the families of F- and V-ATPases that operate without any apparent phosphoenzyme intermediate [2–5]. The vacuolar H^+-ATPase (V-ATPase) is responsible for acidification of the interior of these compartments and for providing the energy for transport processes that take place across their membranes.

In the yeast vacuole the V-ATPase is involved in numerous physiological processes, including metabolite storage and pH homeostasis [6]. For example, seven different amino acid transporters that rely on proton antiport have been biochemically identified in the vacuolar membrane, although none of the genes encoding these permeases have been identified. Recently, the *VCX1* gene encoding a vacuolar H^+/Ca^{2+} antiporter was cloned and sequenced [7]. This exchanger, in conjunction with the vacuolar Ca^{2+}-ATPase encoded by *PMC1* [8, 9], is involved in maintenance of cytosolic Ca^{2+} concentrations.

Due to its location in the organelles of the secretory pathway, V-ATPase also plays a role in protein targeting. Compartment acidification is important for efficient delivery of proteins to the yeast vacuole and mammalian lysosome. Inhibition of V-ATPase with the drug bafilomycin A_1 results in the accumulation and mis-sorting of precursor forms of various vacuolar hydrolases [10]. Similarly, disruption of individual genes encoding V-ATPase subunits results in a mis-sorting phenotype [11, 12]. Most recently a subunit-depletion approach was used to demonstrate that proteins having lumenally oriented targeting signals were directly dependent on compartment acidification for efficient vacuolar delivery [13].

V-ATPase functions not only in the vacuolar system but also in the plasma membrane of certain specialized cells. For example, the kidney plays a vital role not only in cleaning the body of waste products but also in the acid-base balance of mammals [14, 15]. In epithelial cells of the proximal urinary tubule, V-ATPase is present in the apical membrane and functions in proton secretion [16]. In the collecting duct V-ATPase may be found either in apical or basolateral membranes of specialized intercalated cells. These cells shuttle V-

* Corresponding author.

ATPase between intracellular vesicles and the plasma membrane in response to changes in the acid-base balance of the animal. It was shown that the distribution of V-ATPase in apical or basolateral membranes of intercalated cells changes during adaptation to acidosis or alkalosis. The cells increase the number of V-ATPase enzymes in their apical membrane during acidosis and decrease their number during alkalosis. Therefore, V-ATPases play a major role in maintaining pH homeostasis in mammals and other animals, similar to their role in yeast.

Another important role for V-ATPases in regulating pH is seen in the process of bone growth. Bone resorption is necessary for bone growth, remodelling and repair [17]. Osteoclasts are multinucleated and highly motile cells that migrate between the bone and bone marrow, and function in bone resorption. They attach to the mineralized bone matrix forming a closed space into which hydrolytic enzymes are secreted. These enzymes require low pH for optimal activity. This pH level is provided by V-ATPase located in the part of the plasma membrane in contact with the bone [18–23]. The principal bone mineral is hydroxyapatite, and protons are required for the release of each calcium ion from the mineral. The osteoclast V-ATPase provides the protons that are necessary for calcium resorption. As in kidney intercalated cells, osteoclasts shuttle V-ATPase between intracellular vesicles and the plasma membrane in response to attachment onto the bone matrix. This action turns an amoeba-like cell into a polar cell.

In the past decade impressive progress has been made in elucidating the properties, structure and evolution of V-ATPases [1, 5]. Mammalian cells were the primary source for the isolation and purification of V-ATPase from chromaffin granules [24–26], kidney microsomes [27] and clathrin-coated vesicles [28–30]. The conservation of V-ATPases in different eukaryotic cells provides the opportunity to study the biochemistry of the enzyme in mammalian systems and to carry out genetic studies in yeast.

Structure of V-ATPases

Figure 1 shows an SDS-polyacrylamide gel of purified V-ATPase from bovine chromaffin granules. The V-ATPase is a complex modular protein containing several subunits that are divided into catalytic and membrane sectors. The catalytic sector subunits are denoted as subunits A to F, and the membrane sector subunits are marked by the letter M followed by their apparent molecular weight on the gel (fig. 1). The function of the catalytic sector is to provide the ATP binding site and to catalyse the ATP formation and/or ATPase activities of the enzyme. The main function of the membrane sector is to conduct protons across the membrane. The energy coupling between these two processes is believed to be catalysed via mechanochemically induced conformational changes [31]. Figure 2 depicts our current view of the subunit structure of V-ATPases. It is based on studies from several laboratories working on the mammalian and yeast enzymes. It includes subunits that were shown by genetic means (see below) to be necessary for the function and/or assembly of V-ATPase in yeast and are present at significant amounts in the purified enzyme. Not included in figure 2 are some gene products that are necessary for the assembly and/or function of the active enzyme but that are not thought to be part of the final complex [32–36]. The gene names, mammalian subunit designations and deduced molecular weights (kDa) of the yeast subunits are as listed in table 1.

The fine structure of V-ATPases is not known. Crystallization attempts are hampered by the lack of a convenient and abundant source of the enzyme, and by the characteristic that the catalytic sector by itself has no ATPase activity [52, 53]. At present functions have been definitively assigned to only 4 of the 11 subunits that comprise the V-ATPase. In these four cases the functional assignments are based primarily on homology to

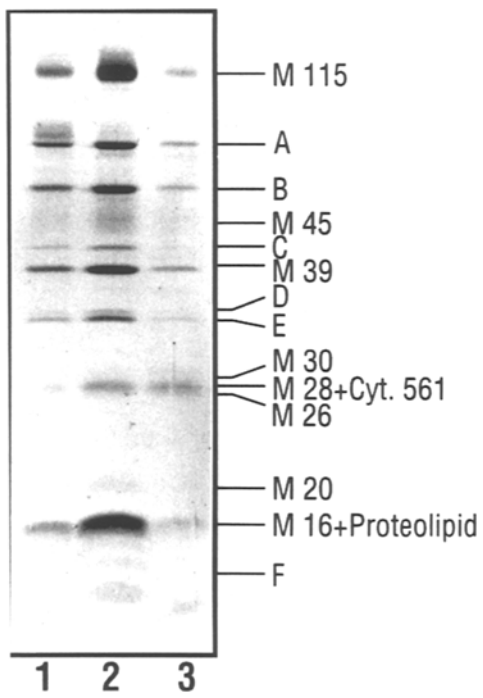

Figure 1. Polypeptide composition of purified V-ATPase from bovine chromaffin granules. The V-ATPase complex was purified from the chromaffin granules by centrifugation through a glycerol gradient. The gradient was fractionated, and the fractions were analysed by electrophoresis on SDS-polyacrylamide gel. The gel was stained with Coomassie blue. Three fractions (1, 2, 3) containing the peak of ATPase activity are shown. In addition to previously identified subunits (M115, A, B, M45, C, M39, D, E, F, M16 and proteolipid), the preparation contains the putative subunit *a* of V-ATPase (M20). The preparation also contains additional polypeptides (M30, M28, M26 and cytochrome 561). While M30, M28 and M26 copurify with the enzyme, cytochrome 561 does not coincide with the fractionation of the V-ATPase.

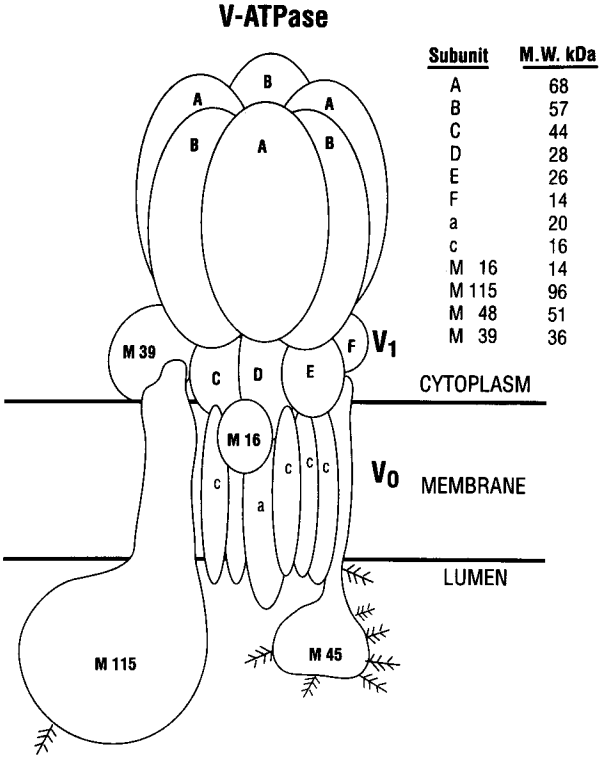

Figure 2. Overall subunit structure of a representative V-ATPase. Subunits A, B, C, D, E and were positively identified, and the cDNAs or genes encoding these subunits were cloned from both mammalian and yeast sources. In the membrane sector, subunits M115, M39, M16 and the proteolipid (subunit *c*) were positively identified in yeast and mammals by cDNA and genomic cloning. Subunit *a* was identified in preparations of V-ATPase from mammalian sources, but the cDNA encoding this subunit was not cloned. M45 represents an organelle-specific accessory subunit. Potential glycosylation sites are indicated by (|).

subunits in the F-ATPases (α, β, *b* and *c*). The remaining V-ATPase subunits have very little or no homology with F-ATPase subunits, making the large body of information available from studies of F-ATPase of limited value in this regard.

The structure of the catalytic sector of F-ATPase from bovine heart mitochondria was recently determined at 2.8 Å resolution [54]. It revealed an asymmetric structure with a 40-Å stem which contains two α helices in a coiled-coil arrangement. This structure presumably comes from the γ subunit and was implicated in the mechanochemical mechanism of ATP-dependent proton pumping and proton motive force (pmf)-driven ATP formation. The ramifications of this structure are far-reaching, and they present a major step forward in understanding energy coupling in ATP-dependent proton pumps. There is an intriguing question of how much of this structure, and consequently the mechanism of action, will be preserved in V-ATPases. The γ subunit has been implicated for a long time in energy coupling between the catalytic and membrane sectors. We think that an analogous structure exists in V-ATPases, and Vma8p is a likely candidate to fulfill the function of energy coupling in these enzymes [47].

Catalytic sector

The catalytic sector (V_1) contains six different polypeptides denoted as subunits A to F that are encoded in yeast by the genes *VMA1*, *2*, *5*, *8*, *4* and *7* respectively. The stoichiometry of subunits A, B, C, D and E in the catalytic sector of V-ATPases from bovine clathrin-coated vesicles was determined to be 3:3:1:1:1, respectively [55]. The catalytic sector of V-ATPase can be removed with mild treatment; incubation of membranes on ice in the presence of MgATP and 0.2 M NaCl results in the removal of the catalytic sector, but no proton leak is generated by this treatment [43, 52, 56, 57]. This suggests that in one of the conformations of the catalytic cycle the contact between the catalytic and membrane sectors is hydrophobic in nature (hydrophobic interactions are weakened at low temperature). This feature of V-ATPase was utilized for several biochemical and bioenergetic studies of V-ATPase from various sources. Biochemical studies of resolution and reconstitution were performed on the catalytic sector of V-ATPase from clathrin-coated vesicles. Stone and his colleagues were able to isolate individual subunits of the catalytic sector of V-ATPase and to reconstitute a calcium-dependent ATPase activity from isolated preparations of four different subunits [36, 58–60]. Moreover, substitution of some of the subunits by recombinant subunits, isolated following overexpression in *Escherichia coli*, resulted in an active enzyme. Forgac and his colleagues were able to isolate and reconstitute the catalytic sector back onto the membrane sector and to study in detail the components necessary for proton pumping activity of the enzyme [53, 61, 62]. These biochemical experiments nicely complemented the genetic studies in yeast mutants that lacked specific subunits [43, 63–66].

The properties and function of the catalytic subunits are as follows:

Subunit A/Vma1p (68 kDa) of V-ATPase is the *N*-ethylmaleimide (NEM)-sensitive catalytic ATP-hydrolysing subunit [25, 67–69]. The amino acid sequence of this subunit contains a glycine-rich motif that is common for ATP-binding proteins [70]. This motif contains two cysteine residues which, when modified, cause inactivation of the enzyme [71]. Moreover, modification of a single cysteine on bovine subunit A prevents dissociation of the catalytic sector from the membrane by cold treatment [52]. These and other observations leave little doubt that subunit A functions in the ATPase activity of V-ATPases by providing the catalytic ATP-binding site, thus making it equivalent to the β subunit of F-ATPase.

Subunit B/Vma2p (57 kDa) of V-ATPase may contain a regulatory ATP-binding site. This subunit binds ATP

Table 1.

Gene	Subunit	MW	Proposed function	Reference
VMA1	A	68	ATP binding, catalytic	37
VMA2	B	57	ATP binding, regulatory	38, 39
VMA3	*c*, Proteolipid	16	Proton translocation	40, 41
VMA4	E	27	?	42
VMA5	C	42	?	43
VMA6	M39	40	?	44
VMA7	F	13	?	45, 46
VMA8	D	28	Energy coupling?	47, 48
VMA10	M16	13	V_1-binding, energy coupling	49
VPH1, STV1	M115	95	?	50, 51

analogs only under restricted conditions [72]. It does not bind ATP following ultraviolet (UV) irradiation [69]. Recently it was shown that subunit B could be labelled by 2-azido-[^{32}P]ATP in the isolated coated vesicle V-ATPase [73], and by ATP or adenosine diphosphate (ADP) following UV irradiation in the isolated recombinant subunit B. Sequence analysis revealed an extensive homology to the α subunit of F-ATPases [74]. Subunit B, however, contains no glycine-rich sequence, normally found in the nucleotide binding site. These and other observations suggest that subunit B may function in regulating the activity of V-ATPase.

The remaining subunits in the catalytic sector of V-ATPase have no homology to F-ATPase subunits. No function has been assigned to subunits C, D, E and F of V-ATPase. Recently, however, we cloned a bovine cDNA and a yeast gene (*VMA8*) encoding subunit D/Vma8p of the respective V-ATPases [47]. Even though no significant sequence homology was found between subunit D and the γ subunit of F-ATPases, structural analysis indicated similar motifs in the two proteins.

Membrane sector

The function of the membrane sector is to conduct protons across the membrane and to couple this vectorial action with the process of ATP formation or hydrolysis. While the membrane sector of *E. coli* F-ATPase consists of three different subunits, that of mammalian mitochondria may contain up to 10 different polypeptides [75, 76]. Similarly, the membrane sector of mammalian V-ATPase may be composed of multiple subunits (see fig. 1).

The proteolipid or subunit *c*/Vma3p depicted in figures 1 and 2 as the 16-kDa polypeptide is the principal subunit of the membrane sectors of V-ATPase. The identification of a *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding site in proteolipids of F-ATPases led to an extensive study of their structure and function [77]. In the F-ATPase this protein of 8 kDa contains about 80 amino acids. It is highly hydrophobic and soluble in a chloroform/methanol solution. Its detailed structure and orientation in the *E. coli* membrane is known [77–79]. The *E. coli* proteolipid is built of two transmembrane helices with a hairpin facing the catalytic sector in the cytoplasm. In the middle of the second transmembrane segment there is a glutamyl or aspartyl residue that provides the binding site for DCCD. DCCD binding blocks proton conductance across the membrane and therefore inactivates the enzyme. The proteolipid of V-ATPase is also a highly hydrophobic protein that binds DCCD, but contains about 160 amino acids (16 kDa) [40, 80]. DCCD binding inactivates the proton pumping and ATPase activities of the enzyme [81, 82]. The cDNA and the gene encoding this subunit in mammals and yeast were cloned and sequenced [40, 80]. The sequences revealed that the proteolipid evolved by gene duplication and fusion of an 8-kDa-encoding ancestral gene homologous to that present in F-ATPase. Disruption of the gene encoding the proteolipid in yeast cells showed that it is necessary for the assembly of all other subunits of the enzyme [44, 63]. The proteolipid is likely to be involved in the process of proton translocation across the membrane.

Recently we discovered a novel subunit of the membrane sector of V-ATPase which is homologous to subunit *b* of the F-ATPase [49, 83]. We identified a protein (M16) that copurifies with the V-ATPase complex from bovine chromaffin granules. Information obtained from the amino acid sequence of a proteolytic fragment of M16 was used to clone a bovine adrenal cDNA encoding this protein. The cDNA encodes a hydrophilic protein of 118 amino acids with a calculated molecular mass of 13,682 Da. Amino acid sequence analysis revealed that M16 exhibits a significant homology to subunit *b* of F-ATPases. A homologous protein was identified in the yeast V-ATPase, and the gene encoding this subunit (*VMA10*) was cloned, sequenced and interrupted [49]. M16 is smaller than subunit *b* and contains no apparent transmembrane segment in its N-terminus. The remainder of subunit *b* is related to M16 not only by its amino acid sequence but also in its predicted structure of helix-turn-helix. The hydrophilic part of subunit *b* of F_0 was identified as part of the stalk structure of the enzyme. A substantial body of evidence suggests that the *b* subunit participates

in the interaction between F_1 and F_0 (see ref. 75). Therefore, the *b* subunit is likely to play a direct role in the transmission of energy derived from ATP hydrolysis to proton translocation across the membrane. Recently, using recombinant DNA methods, the hydrophilic part of the *b* subunit was expressed in *E. coli* [84]. The polypeptide formed a dimer with a sedimentation characteristic indicating an elongated shape and displayed a circular dichroism spectrum suggesting that it is in the α-helix conformation. This evidence is consistent with structural predictions by several computation methods [85]. Consequently, the *b* subunit is viewed as having two long α helices broken by an apparent turn. Structural analysis of the yeast Vma10p V-ATPase subunit indicates an almost identical structure to this subunit [83]. It has a very high tendency to form an α helix, and like subunit *b* of F-ATPase is broken in the middle by an apparent turn. These structural similarities between M16 (Vma10p) of V-ATPase and subunit *b* of F-ATPase suggest that they may play a similar role in the mechanism of action of the respective enzymes.

The remaining subunits in the membrane sector of V-ATPase have no homology to F-ATPase subunits. M115 (Vph1p) is the most unusual of the V-ATPase subunits which have no homology or analogy in F-ATPase. Its function is not known, but it is proposed to play a role in targeting the V-ATPase complex to the vacuole [51]. The protein has a large hydrophilic amino-terminal domain (the topology of this domain is not known) and is predicted to contain six to eight transmembrane regions [50]. M115 (Vph1p) is thought to bind the catalytic sector of the enzyme [50, 65]. A homologous subunit, Stv1p, is proposed to replace Vph1p in the Golgi-resident V-ATPase [51].

M39 (Vma6p) is also a unique subunit of V-ATPase that is associated with the membrane sector but contains no apparent transmembrane segments [86]. It is predicted to interact with vacuolar membranes by direct interaction with other V_0 polypeptides. It may also be involved in binding V_1 [44]. Additional subunits associated with the membrane sector are likely to be discovered. Some of those potential subunits are indicated in figure 1 as M18, 20 and 22. A subunit that will function like subunit α of F-ATPase is still missing, and one of the above-mentioned polypeptides may provide that function.

Molecular genetics of yeast V-ATPase

The presence of an active V-ATPase is necessary for viability of most eukaryotic cells [5]. Energization of the vacuolar system by this enzyme drives several vital transport processes across membranes of the various organelles derived from the vacuolar system [1]. Consequently, it is likely that null mutations in genes encoding V-ATPase subunits will be lethal for most eukaryotic cells. Fortunately, yeast cells survive disruption of various genes encoding V-ATPase subunits [63]. The mutants overcome the lack of acidification of their vacuoles by endocytosis of external fluid [87]. Therefore, the mutants can be grown only at low pH [63]. Under these conditions, however, they cannot overcome high and low calcium concentrations in the medium [41, 64, 88].

With the exception of *VPH1* and *STV1* that encode homologous proteins [50, 51], all genes encoding subunits of V-ATPase are present as a single copy in the yeast genome [47]. Disruption of each of these genes (except for *VPH1* or *STV1*) gave an identical phenotype in which cells could not grow at pH higher than 7 and are sensitive to low and high calcium concentrations in the medium [41, 63, 89]. This phenotype opened the door for site-directed and random mutagenesis studies. The assay for the function of the mutated genes is performed by transforming the respective null mutants with plasmids containing intact or modified genes. Growth at pH 7.5 indicates the presence of a functional gene. The proteolipid (Vma3p) was the first subunit to be studied by site-directed mutagenesis [64]. This study indicated that the proteolipid is quite sensitive to changes in its amino acids, and even some hydrophobic residues that were changed into similar amino acids abolished the activity of the enzyme [64]. The strict conservation of amino acid sequences in potential transmembrane helices suggests tight contacts among the transmembrane segments necessary for activity of the enzyme.

As an initial step in understanding the mechanism of proton transport as well as the membrane topography and interaction of the proteolipid with other subunits, we initiated a study of second-site suppressors that restore activity to an inactive mutant form of the yeast proteolipid [66]. Lack of sufficient charge groups and successful replacements of polar amino acids by glycine in the yeast proteolipid prompted us to suggest that water molecules coordinated to those glycine residues may take part in proton conductance [64]. In helix III of the V-ATPase proteolipid there are five glycines facing the same side of the helix. We mutated each of those glycines to valine residues and found that all these substitutions inactivated the proteolipid [66]. This is in contrast to the overall inactivation rate of about 35% observed in a previous extensive mutagenesis [64]. Therefore, it is apparent that this face of helix III is very sensitive to volume and/or hydrophobicity changes of its amino acid residues. This may be due to tight structural constraints inside each proteolipid monomer, or may indicate that this face of helix III is important for the formation of the proteolipid oligomers [90]. It is worth noting that the effect of substituting these glycine residues was much more deleterious than substitutions in helix IV in the vicinity of E_{137}. A second-site suppres-

sor for the mutation $G_{101}V$ was identified. This suppressor resulted from substituting I_{134} to V, which is located in close proximity to E_{137}. This provides additional support for the notion that the size of the amino acid residues and not their hydrophobicity is the determining factor in inactivation of the proteolipid and in suppression of the inactive mutations.

Recent studies have focused on mutational analyses of genes encoding subunits of the V_1 domain. Forgac and colleagues [91] used site-specific mutagenesis to examine the nucleotide binding sites on subunit B/Vma2p. A mutation of $R_{381}S$ eliminates proton pumping activity and dramatically reduces ATPase activity. This residue is predicted to contribute to the catalytic nucleotide binding site present on subunit A/Vma1p. Glycerol gradient fractionation was used to assess assembly and/or stability of the V_1 complexes resulting from the directed mutations. A partial instability of the peripheral domain resulting from the $R_{381}S$ mutation suggests that R_{381} may form part of a contact site with the A subunit. Random mutagenesis of the *VMA2* gene revealed that mutations of $E_{317}K$ and $E_{355}K$ resulted in loss of interactions between Vma1p and Vma2p based on the yeast two-hybrid system (J. Tomashek and D. Klionsky, unpublished results). In the case of $E_{317}K$, the V_1 complex was still able to assemble on the membrane. Continued mutational analyses will provide further information on the domains of subunit B/Vma2p that are involved in nucleotide binding, interactions with other subunits and assembly of the V_1 domain.

The homology between the V-ATPase Vma1p, 2p, 3p and 10p subunits and the β, α, *c* and *b* subunits of F-ATPase has allowed the assignment of functional properties to the yeast homologues. These biochemical predictions have been supported by the mutational studies that have been conducted. In the case of subunit E/Vma4p there is very little homology with the F-ATPase, so a specific role has not been assigned to this protein. Vma4p is needed for assembly of the remaining V_1 subunits [92, 93]. Random mutagenesis of *VMA4* has generated alleles that allow assembly of the V_1 onto the V_0 but that are nonfunctional; the yeast mutants are unable to grow at pH 7 but have a V-ATPase complex that binds to the membrane in a Vma3p-dependent manner (K. Morano and D. Klionsky, unpublished results). After solubilization, the V-ATPase from these mutants migrates within a glycerol gradient at a position suggesting normal assembly. Interestingly, the V_1 complexes containing altered Vma4p subunits do not appear to be as stable in the cytosol as those complexes assembled with native Vma4p (K. Morano, J. Tomashek, B. Garrison and D. Klionsky, unpublished results). These preliminary data suggest that Vma4p contributes to the catalytic activity of the V_1.

Biogenesis of V-ATPase

In both plant and animal systems, tissue- or developmentally specific isoforms of V-ATPase subunits exist. In yeast, all subunits of V-ATPase are encoded by single genes (with the possible exception of *VPH1*/*STV1* as noted). The yeast system is therefore ideal for carrying out an organized and systematic mutational analysis to determine the properties of individual ATPase subunits and their contribution to the intact enzyme complex. Most genes encoding structural proteins of V-ATPase have been cloned, sequenced and disrupted [47]. Initial studies with null mutants showed very clearly that each of the V-ATPase subunits is required for the proper assembly of the complex [35, 43, 44, 64]. In general, all of the V_0 subunits are required for assembly of the V_1 onto the membrane. For example, interruption of the gene encoding the proteolipid prevented the assembly of all the subunits of the catalytic sector onto the membrane sector. Recent data, however, indicate that partial complexes of V_1 subunits can form in the cytoplasm in the absence of an assembled V_0 domain [93, 94]. We have developed a native gel electrophoresis system that allows a finer resolution of cytosolic V-ATPase complexes than is possible with glycerol gradients. Using this system we have detected a major cytosolic V_1 complex of approximately 576 kDa present in wild type, *Δvma3* and *Δvma5* strains. Strains having mutations in genes encoding the Vma1p, 2p, 4p, 7p or 8p V_1 subunits fail to assemble this complex, but in some cases reveal large intermediate complexes; for example, we detect a 317-kDa complex containing Vma1p, 2p, 7p and 8p in the *Δvma4* strain and a complex of approximately 96 kDa which appears to contain only Vma4p and Vma10p in all strains except the *Δvma4* and *Δvma10* (J. J. Tomashek and D. J. Klionsky, unpublished results). These results suggest a key role for Vma4p in the final assembly steps to a complete V_1 complex.

As indicated earlier and from the above data, Vma4p is required for assembly of V_1 onto V_0 and for the interaction of Vma1p and Vma2p with other peripheral subunits [44, 92]. The 42-kDa Vma5p is needed for assembly of the holoenzyme but the remaining V_1 subunits can form a complex in the absence of this subunit [32, 65, 92, 93]. This is similar to the 40-kDa subunit from the clathrin-coated vesicle V-ATPase [95]. The Vma8p subunit is required for assembly of the V_1 and association with V_0 [47, 48]. None of these V_1 subunits is required for assembly of the integral membrane V_0; interruption of genes encoding subunits of the catalytic sector did not interfere with the insertion of the proteolipid into the vacuolar membrane [43]. In contrast, Vma7p (subunit F) has properties which make it similar to both V_1 and V_0 subunits. Vma7p is a hydrophilic protein that fractionates with the peripheral domain

[45, 46]. This subunit is the only V_1 protein that is required for assembly of the V_0; in a *Δvma7* strain there is a dramatic decrease in the levels of the V_0 subunits in vacuolar membranes [46].

The VMA3 gene product plays a central role in V-ATPase assembly, and none of the remaining V_0 subunits assemble in the absence of Vma3p [44, 96]. The Vma6 and Vma10 proteins both associate with the membrane but have no apparent transmembrane domains [44, 49]. Both subunits are needed for assembly of the V_1 onto the membrane, but only Vma6p is required for membrane association of the other V_0 subunits. As indicated earlier, Vph1p and the homologous Stv1p may be involved in targeting the enzyme to specific organelles [50, 51]. Vph1p is required for assembly of the V_1 onto the vacuole membrane.

New genes were discovered encoding proteins that are not a part of the complex but that affect its assembly [33, 34, 36, 97]. Among them, Vma21p is a very interesting integral membrane protein of 8.5 kDa [36]. It was shown that this protein resides in the endoplasmic reticulum (ER). It was suggested that Vma21p is required for the assembly of the membrane sector of the V-ATPase in the ER. Moreover, the unassembled 96-kDa integral membrane subunit (Vph1p) is rapidly degraded in the mutant lacking Vma21p. The 21-kDa Vma22 protein is also an ER-localized protein required for V-ATPase assembly [97]. As with Vma21p, the absence of Vma22p results in degradation of Vph1p and prevents the assembly of V_1 onto the membrane. The association of Vma22p with the ER is itself dependent on another 'assembly factor', Vma12p (25 kDa). Again, mutants defective in Vma12p have lower levels of V_0 subunits in the vacuole membrane and fail to assemble the peripheral sector (34). The *VMA11* gene has a high level of identity with *VMA3*, suggesting that the 17-kDa Vma11p may be another proteolipid-like protein [33]. The Vma11 protein, however, has not been localized within the cell. Vma11p is required for assembly of the V_1 onto the V_0 and assembly and/or stability of the remaining V_0 subunits. These results suggest that the V_0 assembles in the ER and is subsequently transported to other locations within the cell. In the absence of correct assembly, the integral membrane domain may be degraded at the level of the ER. These studies are important first steps in an understanding of the way in which the membrane sector is assembled and segregated to the correct locations within the yeast cell.

Coming back to the mammalian system, we searched for organelle-specific proteins in purified preparations of V-ATPase from bovine chromaffin granules. A diffused protein band at a position of about 45 kDa was identified in SDS-polyacrylamide gels of the above preparation. Following digestion with endopeptidase Glu-C (V8), a polypeptide of about 10 kDa was isolated and subjected to amino acid sequencing. The cDNA encoding the protein M45 was cloned from a bovine adrenal medulla library [98]. The cDNA sequence contains an open reading frame encoding a protein of 468 amino acids with a calculated molecular weight of 51,786 Da. A potential signal sequence composed of the first 35 amino acids and a potential transmembrane domain at the C-terminal of the protein were identified. To obtain more information on the localization of M45, we raised a specific antibody against a recombinant fusion protein. Western blot analysis indicated that the M45 polypeptide copurified with the V-ATPase from chromaffin granules. Immunocytochemical staining of sections from bovine adrenal glands revealed intense staining in the medulla and almost no staining in the cortex. We suggest that membrane proteins analogous to M45 may function in specific organelles as a template for the assembly of V-ATPase as well as modulators of its activity.

Expression of cDNAs encoding heterologous V-ATPase subunits in yeast

In both plant and animal systems, tissue- or developmentally specific isoforms of the V-ATPase subunits exist. These subunits may have unique functions in regulating the localization and/or activity of the enzyme. Yeast provides the best-known system for functional studies of organelle-specific subunits. One can readily interrupt a gene encoding an individual subunit in yeast and try to complement the phenotype resulting from this disruption by expressing specific mammalian cDNAs encoding specialized V-ATPase subunits. The first step towards this goal was achieved by the demonstration that a chimeric yeast/bovine gene encoding Vma5p/subunit C successfully complemented the phenotype of the interruption mutation *Δvma5::LEU2* [43]. A similar approach was taken for other cDNAs encoding bovine subunits. Recently it was reported that cDNAs encoding subunits B and D can be successfully expressed in yeast and complement the phenotype of the corresponding disruption mutations [47, 99]. We undertook a similar approach to analyse the Vma4 and Vma5 proteins. We constructed a chimera from the *VMA5* gene encoding Vma5p of yeast and cDNA encoding bovine subunit C. The chimeric gene was inserted into the yeast genome by transforming the yeast mutant *Δvma5::LEU2* with a linear DNA encoding the chimera. The transformed yeast cells were grown on YPD-agar plates buffered at pH 7.5 [63]. Colonies that grew under these conditions were analysed for growth on minimal plates with or without leucine. Most of the above-selected colonies failed to grow on plates without leucine, indicating that the interrupted *VMA5* gene was displaced by the chimeric gene in the yeast genome [100]. This observation was confirmed by polymerase chain reaction (PCR) experiments using specific

oligonucleotides for the bovine cDNA and the flanking regions of the yeast gene.

Similarly, the *VMA4* gene of this strain (having the Vma5p/subunit C chimera in the genome) was interrupted by introducing the *LEU2* gene inside its reading frame. This resulted in a phenotype where once again the cells could not grow at pH 7.5 [42]. This phenotype could be complemented by transforming the mutant cells with a plasmid carrying the *VMA4* gene. A chimeric gene of Vma4p/subunit E was constructed such that only a third of the gene encodes the DNA sequence obtained from the bovine cDNA (L. Supekova and N. Nelson, unpublished). Remarkably, the chimeric Vma4p/subunit E gene failed to complement the phenotype resulting from the mutation *Δvma4::LEU2* in the yeast double chimera strain. Suppressor mutations to this inactive chimeric gene were obtained by growing the transformed yeast in a medium buffered at pH 6.7 for several generations. Then the cells were streaked on 1% yeast extract, 2% bactopeptons, 2% glucose and 2% agar (YPD) plates buffered at pH 7.5. Single colonies that grew well at pH 7.5 were analysed by isolating their plasmids and transforming the disruption mutant *Δvma4::LEU2*. Analysis of the plasmids that complemented the phenotype revealed that they contain a mutated DNA in the chimeric gene in which D_{31} was substituted to Y, thus rendering the chimeric gene active. In essence, one lets the microorganism give the correct answer of what is wrong with a given inactive gene.

The experiments described above indicate that expression of mammalian cDNAs encoding V-ATPase subunits is feasible and potentially useful for analysing their specific properties. Using the same system for the study of plant V-ATPase subunits is also quite feasible. By combining molecular genetic approaches that are possible in yeast with the analysis of tissue- and developmentally specific isoforms of V-ATPase subunits from plant and animal cells, we will be able to make rapid advances into understanding the structure, function and regulation of this ubiquitous, complex and fascinating enzyme.

Concluding remarks

Yeast cells contain most of the fundamental organelles, metabolic pathways, signal transduction processes and checkpoints of eukaryotic cells. Their convenient genetics, together with the sequencing of the complete yeast genome, make them an ideal system for the study of fundamental life processes. Understanding conserved fundamental systems is a prerequisite for understanding complex processes in mammalian cells. Studies on mitochondrial biogenesis provide a prime example of this approach [101]. Jeff Schatz introduced me (N.N.) to the yeast system and enlightened me about the advantages of their genetics combined with advanced biochemical and immunological studies [102]. We adopted this philosophy in the study of V-ATPases and made an early switch from studies of the biochemistry of the chromaffin granule enzyme to the molecular biology of V-ATPase from yeast cells. We believe that this approach will not only reveal the mechanism of action of this fundamental enzyme but also provide an ideal system for the study of plant and mammalian proteins that confer unique properties to the enzyme isolated from different cells and organelles.

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