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A Journey From Mammals to Yeast With Vacuolar H^+-ATPase (V-ATPase)

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The vacuolar H^+-ATPase (V-ATPase) is one of the most fundamental enzymes in nature. It functions in almost every eukaryotic cell and energizes a wide variety of organelles and membranes. V-ATPase has a structure and mechanism of action similar to F-ATPase and several of their subunits probably evolved from common ancestors. In eukaryotic cells, F-ATPase is confined to the semiautonomous organelles, chloroplasts and mitochondria, which contain their own genes that encode some of the F-ATPase subunits. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form ATP at the expense of the protonmotive force (pmf), V-ATPases function exclusively as ATP-dependent proton pumps. The pmf generated by V-ATPases in organelles and membranes of eukaryotic cells is utilized as a driving force for numerous secondary transport processes. It was the survival of the yeast mutant without the active enzyme and yeast genetics that allowed the identification of genuine subunits of the V-ATPase. It also revealed special properties of individual subunits, factors that are involved in the enzyme's biogenesis and assembly, as well as the involvement of V-ATPase in the secretory pathway, endocytosis, and respiration. It may be the insect V-ATPase that unconventionally resides in the plasma membrane of their midgut, that will give the first structure resolution of this complex.

KEY WORDS: V-ATPase; F-ATPase; protonmotive force; slip; membrane energization; biogenesis; assembly.

PROLOGUE

I was asked to highlight my contribution to the study of V-ATPase during the last 20 years. As I read some recent reviews on the subject, I came to realize that this may be a daunting task as I have apparently made only a minor contribution to the field (Nishi and Forgac, 2002). The following review may therefore be considered as a well-documented, contemporary scientific illusion. So, without any pretense of objectivity, the following is my view on the past, present, and future of V-ATPase research.

The bioenergetic definition of V-ATPase originated from studies with chromaffin granules from bovine adrenal medulla (for review see Nelson and Harvey, 1999). It began with studies on the energization of the catecholamine uptake into chromaffin granules (Kirshner, 1962; Njus and Radda, 1978). In these pioneering studies, it was demonstrated that an ATPase energizes the chromaffin granule membranes by acting as an ATP-dependent proton pump, and that the resulting proton gradient drives the accumulation of catecholamines by exchange for protons (Johnson *et al.*, 1982; Johnson and Scarpa, 1976; Kanner and Schuldiner, 1987). Subsequently, it was demonstrated that a similar proton pump operates in the vacuoles of fungi and plants (Bowman and Bowman, 1982; Kakinuma *et al.*, 1981; Sze, 1985; Uchida *et al.*, 1985). Even though biochemical research identified the enzyme as the V-ATPase (Cidon and Nelson, 1983; Forgac *et al.*, 1983; Stone *et al.*, 1983), studies of the yeast enzyme paved the way for detailed molecular biology analysis of its subunit structure and properties (Nelson and Nelson, 1990). The cloning of genes encoding V-ATPase subunits provided the first evidence that the F- and V-ATPases are related and have evolved from a common ancestor (Bowman *et al.*, 1988b,c; Mandel *et al.*, 1988; Nelson, 1992; Zimniak *et al.*, 1988).

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BIOCHEMISTRY

When the first attempts to isolate the V-ATPase were initiated, the study of membrane biochemistry was in its infancy and molecular biology was practically not available. Resolution and reconstitution of membrane proteins championed by Efraim Racker were practiced on very few systems such as mitochondria and chloroplasts and most of the reconstituted membrane proteins were not of high purity (see Racker, 1976). It was therefore a landmark achievement when Racker and Stoeckenius achieved reconstitution of photophosphorylation by two relatively pure membrane components: bacteriorhodopsin and mitochondrial F-ATPase (Racker and Stoeckenius, 1974). F-ATPase, which operates without the involvement of a phosphoenzyme intermediate was shown to be composed of several subunits assembled into a membrane complex of about 500 kDa. At the same time, some other laboratories studied H^+, Na/K, and Ca ATPases that operate via phosphoenzyme intermediates and are composed of a major membrane protein of about 100 kDa (Serrano *et al.*, 1986). So, when the first attempts to isolate the V-ATPase were carried out, the two structures were dominating the minds of the researchers and indeed, instead of V-ATPase, the F-ATPase or P-type ATPases were isolated (Apps and Schatz, 1979; Roisin and Henry, 1982; Xie *et al.*, 1984). In 1983 we identified the V-ATPase from bovine chromaffin granules as a novel enzyme that is distinct from the other known proton pumps (Cidon and Nelson, 1983). Only in the late 1980s was isolation of partially purified V-ATPases from clathrin-coated vesicles, kidney membranes, and chromaffin granules achieved (Arai *et al.*, 1987; Gluck and Caldwell, 1987; Moriyama and Nelson, 1987; Xie and Stone, 1986). What were the obstacles that prevented the early attempts to obtain the purified enzyme? Essentially, the instability of the enzyme in the cold was not appreciated. Only after the discovery of its unique cold inactivation, which was noticed because of a very rare, long "lunch-break" of Yoshinori Moriyama during which the preparation was left on ice, could the stabilizing conditions for the enzyme be determined (Moriyama and Nelson, 1989a). When the activity of V-ATPase is lost, unrelated ATPases were isolated including a P-type ATPase from chromaffin granules, which later was found to be a phospholipid flippase (Dean *et al.*, 1984, 1986; Moriyama and Nelson, 1988; Zachowski *et al.*, 1989). Further biochemical studies have defined the V-ATPase as a multisubunit protein complex with distinct membrane and catalytic sectors (for review see Stevens and Forgac, 1997). As in the early days of F-ATPase, the number and identity of the various subunits of V-ATPase was subject to the investigator's imagination. It was reported that the purified enzymes from plant and fungal sources contain three subunits of about 70, 60, and 17 kDa (Mandala and Taiz, 1986; Randall and Sze, 1986; Uchida *et al.*, 1985). The reported subunit structure of the enzyme from mammalian sources varied from 6 for bovine chromaffin granules (Cidon and Nelson, 1983; Moriyama and Nelson, 1987), 8–9 subunits in clathrin-coated vesicles (Arai *et al.*, 1987; Xie and Stone, 1986) to 13 for kidney (Gluck and Caldwell, 1987). We all neglected genuine subunits or detected irrelevant polypeptides. Although those preparations were what the biochemists call "reasonably pure," biochemical methods always faced difficulties in the positive identification of a polypeptide as an integral subunit of a membrane protein complex. A genuine subunit must be present in stoichiometric amounts and must be necessary for the complex activity or for the assembly and stability of the holoenzyme. Purified biochemical preparations may contain irrelevant polypeptides or lack genuine subunits that were dissociated during the preparation of the complex, without a detectable effect on its assayed activity. This, like many others, is an example of the need for molecular biology to reach a clear-cut answer. Yeast was the organism that provided the V-ATPase null mutants and has had a major impact on the advancement of our research (Nelson and Nelson, 1990). Yeast genetics also permitted the identification of special properties of individual subunits and the discovery of factors that are involved only in the enzyme's biogenesis and assembly, but nevertheless are obligatory for its activity (Graham *et al.*, 1998).

The biochemical and genetic data are integrated into Fig. 1 to depict the subunit structure and function of V-ATPases in comparison to those of F-ATPase. It is assumed that the function of the homologous subunits is similar in F- and V-ATPases. However, b subunit of F-ATPase is a typical F_0 subunit, whereas the homologous G subunit of V-ATPase was reported to be associated both with V1 and V_0 sectors (Crider *et al.*, 1997; Lepier *et al.*, 1996; Supekova *et al.*, 1995, 1996). Subunit γ of V-ATPhase is likely to be analog of the F-ATPase subunit δ (Nelson *et al.*, 1995), and subunit F as suggested to represent the analogue of ϵ. We need a high-resolution structure of V-ATPase to varify those hallucinations. The problem is that, in most cells, the amount of the V-ATPase is minute and the purification very difficult. One exception is the larval Manduca sexta where the V-ATPase resides in the plasma membrane of the midgut and there comprises the majority of the membrane proteins, thus providing a rich source for its purification (Radermacher *et al.*, 2001; Wieczorek *et al.*, 2000).

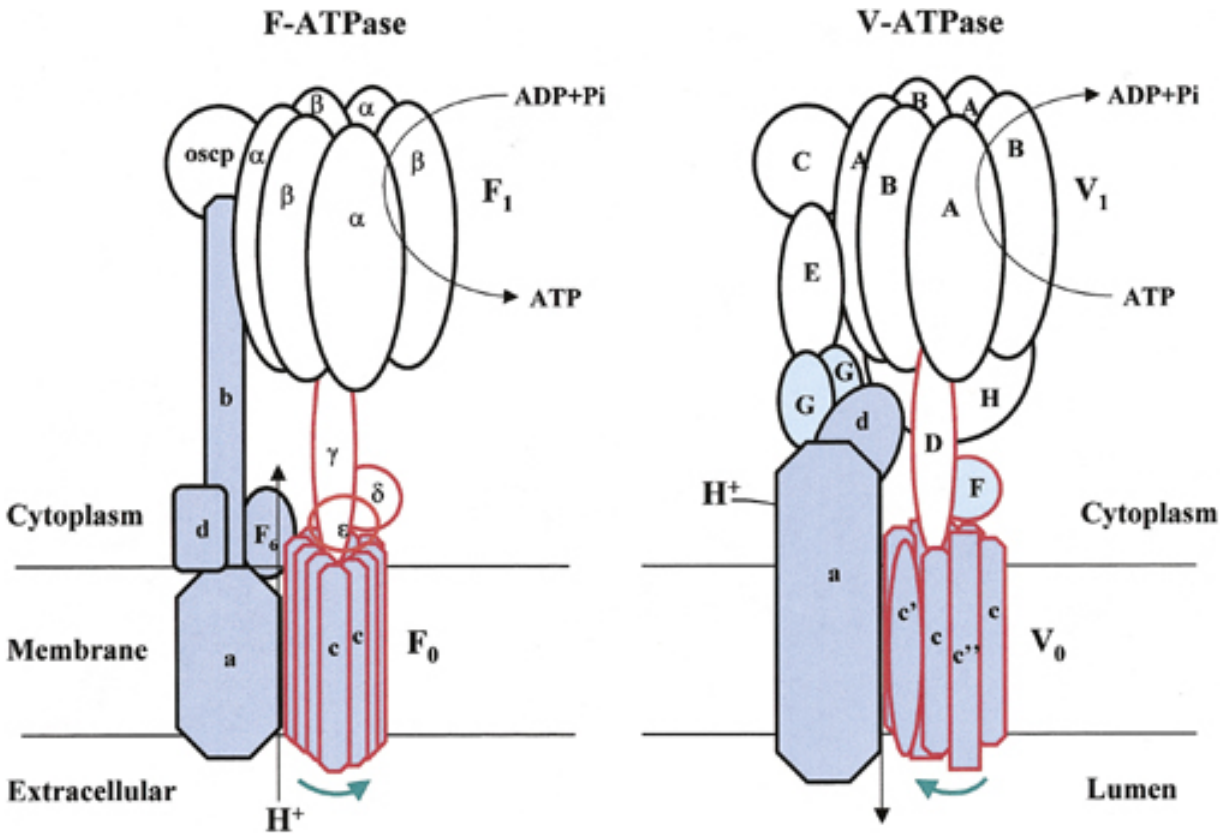

Fig. 1. Schematic depiction of the subunit structure of F- and V-ATPases. Left, subunit composition of mitochondrial F-ATPase (Gibbons *et al.*, 2000); according to Stock *et al.* , 2000 right, yeast V-ATPase. The subunits of the membrane sectors F_0 and V_0 are painted blue and those of the catalytic sectors F1 and V1 are white. Rotating subunits are bordered by a red color, and the green arrow indicates the directionality of rotation.

MOLECULAR BIOLOGY AND GENETICS

Biochemistry of V-ATPase generated the initial knowledge about the enzyme and also provided the foundation for its molecular biology. Today we look down on the gene race of the 1980s, but the ones that participated in it experienced a lot of excitement. In 1987 we had a Bioenergetics Meeting (family style) in Osnabrück. The participants will never forget this meeting because the first sequences of V-ATPase subunits were presented and painted a clear picture of the evolution of V-ATPase from archae to mammals in relation to the evolution of F-ATPase (see Nelson, 1989, 1992, for reviews). All the genes or cDNAs were cloned via reverse genetics from subunit-specific amino acid sequences that were obtained directly from isolated V-ATPase subunits or by screening with subunit-specific antibodies (Bowman *et al.*, 1988b,c; Denda *et al.*, 1988; Mandel *et al.*, 1988; Manolson *et al.*, 1988; Zimniak *et al.*, 1988). The rate-limiting steps in the cloning process were the small amounts of V-ATPase, the need for relatively large protein amounts for amino acid sequencing, and the poor cDNA libraries that were available at that time. Very early in the game, we realized that the gene race in higher organisms must end quite soon after its start, because at that time it was not possible to study mutants in those systems. Therefore, the first library of bovine adrenal medulla cDNA that was constructed in our laboratory was cloned into a yeast shuttle vector that we developed (Wang *et al.*, 1989). The first to be "fished" from this library was the cDNA encoding the Ac39 subunit that is now termed Vma6 or subunit d. Several other cDNAs encoding subunits C, D, G and Ac45 were also cloned from this library (Nelson *et al.*, 1990, 1995; Supek *et al.*, 1994a; Supekova *et al.*, 1996).

Our first step in yeast molecular biology was the cloning of two genes encoding subunits B and c (proteolipid) that were known from plant and mammalian sources, respectively (Mandel *et al.*, 1988; Manolson *et al.*, 1988; Nelson *et al.*, 1989; Nelson and Nelson, 1989). The relative ease in cloning of the yeast genes, based on the available plant and mammalian sequences, indicated that V-ATPase is very conserved even in transmembrane

segments that are usually less conserved. We assumed that null mutations in genes encoding V-ATPase subunits are likely to be lethal for all eukaryotic cells, because primary energization of the vacuolar system by this enzyme drives vital secondary transport processes across membranes of vacuolar-derived organelles (Mellman *et al.*, 1986; Nelson and Klionsky, 1996). Nevertheless we attempted to inactivate genes encoding V-ATPase subunits in yeast cells, in the hope of finding conditionally lethal mutants that would open the door for advanced molecular biology and genetic studies of this enzyme. The appropriate DNA constructs were prepared and the diploid yeast strain was transformed. What to do next? I recalled that Tom Fox is not only an excellent scientist but also likes to perform tetrad dissection by himself. Following a phone call, the cells were sent to Cornell University, they were sporulated and dissected by Tom, and two weeks later, he sent the plate. Only two spores of each tetrad gave colonies, but after additional incubation (forgotten in the cold room) the other two slowly grew to our delight. We discovered that V-ATPase null mutants in yeast have a conditionally lethal phenotype that exhibits growth arrest in buffered medium at neutral pH but grows quite well at pH 5.5 (Nelson and Nelson, 1990). We concluded that yeast V-ATPase null mutants can survive the lack of acidification by taking up acidic external fluid via endocytosis, and indeed the double mutation was lethal (Munn and Riezman, 1994). Ten years later, we also found out that the poor assembly into the plasma membrane of Pma1p in the V-ATPase null mutants is probably the reason for the mutant's very slow growth in lower than pH 5 medium (Perzov *et al.*, 2000). Other features of the restrictive growth of the mutants were discovered later, and actually even now we still explore them to explain the various pathways in which the V-ATPase is involved.

With the exception of *VPH1* and *STV1*, which encode homologous proteins (Manolson *et al.*, 1992, 1994), all the genes encoding V-ATPase subunits are present as a single copy in the yeast genome (Nelson and Harvey, 1999). Disruption of any one of the single-copy genes yields an identical phenotype in which cells cannot grow at a pH higher than 7, and are sensitive to low and high calcium concentrations in the medium (Foury, 1990; Nelson and Nelson, 1990; Noumi *et al.*, 1991; Umemoto *et al.*, 1990). The proteins that are present in stoichiometric amounts, and are encoded by genes whose interruptions lead to this phenotype, must be valid subunits of the V-ATPase. Thus, yeast genetics helped to sort out the genuine V-ATPase subunits and led to the discovery of some novel subunits, as well as proteins that function exclusively in the assembly of the enzyme but are not its genuine subunits (Graham *et al.*, 1998).

Foury (1990) was the first to use the above criterion for arguing that the gene VMA4 encodes the subunit E of V-ATPase. We used it as a criterion for cloning the yeast genes encoding subunits C, D, F, and G (Beltrán *et al.*, 1992; Nelson *et al.*, 1994, 1995; Supekova *et al.*, 1995). The latter contains a story. In all the preparations of purified V-ATPase from yeast vacuoles, a major highly stained band at about 16 kDa on the SDS gels was detected. It appeared to be too prominent to be a "genuine V-ATPase subunit" because of stoichiometric considerations. After we thought that we exhausted the "race" for genes encoding V-ATPase subunits, I decided to look at the stubborn "contamination." The strong band was easily isolated, digested, and a few resulting peptides were sequenced (Supekova *et al.*, 1995). A search in GenBank identified a distinct DNA sequence that had no annotation because it was one of a few yeast genes that contained an intron right after the ATG for the initiator methionine. Following the tradition in our laboratory, the cDNA encoding this subunit (that was later denoted as subunit G) was also cloned from our bovine adrenal library by homology, and it became clear that the suspected contamination was a genuine subunit of V-ATPase. Not only was it a legitimate V-ATPase subunit but also one of very few subunits that are homologous to the corresponding one in F-ATPase. By alignment we also depicted its homology to the b subunit of the membrane sector of F-ATPase (Supekova *et al.*, 1995, 1996). The yeast V-ATPase null mutant became a litmus test for genuine subunits of the V-ATPase complex. When we cloned the Ac39 from bovine chromaffin granules (Wang *et al.*, 1989), it was not legitimate until the yeast VMA6 gene gave it credence (Bauerle *et al.*, 1993). Ideally this system could be used for obtaining functional expression of mammalian V-ATPase subunits in yeast null mutants. Unfortunately we failed to obtain meaningful results in this project, but others were more successful (Hagai *et al.*, 2000; Jones *et al.*, 1995).

The conditional lethality in yeast V-ATPase null mutants also paved the way for extensive molecular biology studies and site-directed mutagenesis of individual subunits (Noumi *et al.*, 1991). Moreover, it enabled the more informative approach of selecting suppressor mutants for inactive mutations (Supek *et al.*, 1994b). The proteolipid (Vma3p) was the first V-ATPase subunit to be studied by site-directed mutagenesis (Noumi *et al.*, 1991). Only 24 of 65 substitutions resulted in inactive proteolipids that could not support growth at pH 7.5. Second-site suppressors were generated by chemical mutagenesis for some of the inactive mutations (Supek *et al.*, 1994b). When the second-site mutations that suppressed the Q90K mutation, for instance, were analyzed, all the mutations were

found to be intragenic and to exhibit space-filling properties, which points to a tightly packed structure. During the search for suppressor mutants generated by EMS treatment, we stumbled onto a big surprise that on one hand hampered our progress, but on the other prompted us to extend our study to cell biology. Once again we were helped by the wisdom of Efraim Racker who coined the expression: "Troubles are good for you" (Racker, 1976).

CELL BIOLOGY AND PHYSIOLOGY

V-ATPase null mutations are pleiotropic and affect several other cellular processes. A pet^- phenotype that cannot grow on a nonfermentable carbon source is one of the consequences of V-ATPase null mutations (Ohya *et al.*, 1991). Later it was demonstrated that the concentration of the nonfermentable carbon source is critical for the vitality of the mutants, and at low concentrations their growth is normal (Supek *et al.*, 1994b). Sorting of secretory proteins in the Golgi is also affected by the lack of V-ATPase activity (Nelson and Nelson, 1990; Perzov *et al.*, 2002; Umemoto *et al.*, 1990; Yaver *et al.*, 1993). Strains with disruptions of the genes encoding the A, B, and c subunits of the V-ATPase accumulate precursor forms of the vacuolar membrane protein, alkaline phosphatase, and the soluble vacuolar hydrolases, carboxypeptidase Y and proteinase A (Yaver *et al.*, 1993). In V-ATPase null strains, the intracellular precursors of the above-mentioned proteins accumulate in the secretory pathway at some point before delivery to the vacuole, but after transit to the Golgi complex. Thus, V-ATPases are intimately involved in the secretory pathway but their absence does not shut down the system and therefore, the growth arrest of the V-ATPase null mutants cannot be attributed to an interference with biogenesis processes in this pathway. V-ATPase is also intimately involved in endocytosis and receptor recycling (Nelson and Harvey, 1999). Here too, the absence of V-ATPase activity cannot explain the cause of lethality in eukaryotic cells with an inactive enzyme. V-ATPase is also involved in the correct assembly of Pma1p into the plasma membrane of yeast cells. Null mutations in V-ATPase subunits caused a marked reduction in the amount of Pma1p in the plasma membrane and large accumulation of an inactive transporter in the endoplasmic reticulum (Perzov *et al.*, 2000). Therefore, it is apparent that V-ATPase is involved not only in the energization of the vacuolar system but also in other cellular aspects. However, V-ATPase is not absolutely required for all the effects we listed above. Since except for Saccharomyces cerevisiae no other organism can tolerate null mutations in V-ATPase, the discovery of bafilomycin A as a specific inhibitor of V-ATPase was of major importance (Bowman *et al.*, 1988a).

Because of the vital requirement for V-ATPase in eukaryotic cells, we were surprised to discover that, following EMS mutagenesis of yeast V-ATPase null mutants, numerous colonies could grow at pH 7.5 (Cohen *et al.*, 1999). Several complementation groups were responsible for the svf (*s*uppressor of *V*-ATPase *f*unction) phenomenon but only one gene (*VTC1*) was so far identified. The *svf* mutations are recessive, and in the presence of the corresponding intact gene in a diploid cell, they failed to grow at pH 7.5. Obviously, the V-ATPase is totally inactive in the *svf* suppressor mutants. *VTC1* is one out of four homologous genes (*VTC1-4*) that encode membrane chaperons, probably helping in the distribution of membrane proteins including V-ATPase (Cohen *et al.*, 1999). *VTC1* was cloned from amino acid sequence information obtained from isolated yeast V-ATPase. It comigrated onto SDS gels with Vma10p, copurified with V-ATPase, and was estimated to be present in stoichiometric amounts with the enzyme (Cohen *et al.*, 1999). Studies that followed the discovery of this gene family showed that the various Vtc proteins form complexes among themselves, and with the V-ATPase are also involved in key aspects of membrane fusion reactions (Müller *et al.*, 2002; Peters *et al.*, 2001). Yeast is a very sturdy organism: In many instances when one system shuts down because of a null mutation of a major protein, another backup system comes to life. Might it be that, in null V-ATPase mutants, backup fusion systems sprout in the *svf* mutants and enable their growth on pH 7.5 medium? Might the activity of V-ATPase in the fusion process be the most crucial one? Discovering the function of the other Svf proteins may shed light on an involvement of V-ATPase in these key processes.

The membrane sector (V_0) of V-ATPase consists of at least five subunits (*a*, *c*, *c′*, *c″*, and *d*) and functions in proton translocation (Nelson and Harvey, 1999). Only subunit a is encoded by two genes (*VPH1* and *STV1*) in the yeast genome. The two genes encode two isoforms of a subunit (Vph1p and Stv1p), and only disruption of both subunits results in the null V-ATPase mutant phenotype (Manolson *et al.*, 1992, 1994). Vph1p was assigned to the vacuole and Stv1p to Golgi and/or endosomes (Kawasaki-Nishi *et al.*, 2001; Perzov *et al.*, 2002). These observations could suggest that subunit a may be responsible for the V-ATPase localization in specific subcellular compartments and/or regulation of enzymatic activity. This implies that there should be specific and distinct phenotypes for both mutants. While the *vph1*Δ mutant is unable to accumulate quinacrine in the vacuole, and therefore appears to be defective in vacuolar acidification, no phenotype was described for loss of Stv1p, and the *stv1*Δ mutant

was reported to be identical to the wild-type (Manolson *et al*., 1994). We analyzed the intracellular localization of Stv1p using a rabbit polyclonal antiserum against a bacterially expressed fragment of Stv1p (Perzov *et al*., 2002). We concluded that Stv1p compensates for the loss of Vph1p in the *vph1*Δ strain by increasing the stability or overproduction of Stv1p. On sucrose gradients the Vph1p profile matches that of the Pep12p profile, suggesting that it is located in the endosomes. Stv1p is located in two peaks along the gradient that coincided with Sec 14p (*trans* Golgi marker), and the second peak matched Pep12p and Sed5p, suggesting its different location from Vph1p.

During this study we discovered that the acidotropic probe LysoSensor Green DND-189 that was used in plants and mammalian cells, is an excellent marker for the acidification of the yeast vacuolar system (Cousin and Nicholls, 1997; Guttenberger, 2000). The dye labeled the vacuolar membranes of wild-type yeast cells in less than 5 min, having a signal that remained stable for over an hour, and was more efficient than the staining with quinacrine. The vacuolar membrane was stained in wild-type yeast cells but not in V-ATPase null mutants (Perzov *et al.*, 2002).

The relationship between the V-ATPase function and the endocytosis processes is intriguing. Endocytosis is responsible for the viability of yeast V-ATPase null mutants on the one hand, while some of the endocytosis mutants were described to have a very similar phenotype to the V-ATPase null mutant on the other (D'Hondt *et al.*, 2000; Munn and Riezman, 1994; Yoshida and Anraku, 2000). In mammalian cells, the acidification of endosomal compartments is important for several endocytic processes (Mellman *et al.*, 1986). Endosomal carrier vesicle formation, as well as transfer between late endosomes and lysosomes, are pH-sensitive processes inhibited by the specific V-ATPase inhibitor bafilomycin A (Clague *et al.*, 1994; van Deurs *et al.*, 1996 van Weert *et al.*, 1995). Indeed, recently the proteolipid Vma3p and the Vtc proteins were found to participate in membrane fusion complexes (Müller *et al*., 2002; Peters *et al.*, 2001). Also the internalization of the dye FM 4-64 in the yeast V-ATPase null mutants was inhibited (Perzov *et al.*, 2002). The Vph1p null mutant displayed the same phenotype as V-ATPase null mutants, but the Stv1p null mutant had similar staining kinetics by FM 4-64 to wild-type cells. We conclude that the V-ATPase function not only determines the pH conditions in the lumen of their respective organelles, but the complex is also connected with intervesicular activities involved in membrane fusion such as those of endocytosis. The latter may be only one of several direct interactions of V-ATPase subunits with cellular processes.

BIOENERGETICS

There are two challenging bioenergetic properties of V-ATPase that attracted our attention in the past and should be properly addressed in the future. One is the apparent lack of proton conductivity across the membrane sector in the absence of the catalytic sector. The second is the inability of most eukaryotic V-ATPases to reach thermodynamic equilibrium.

Several protein isolation procedures call for working at cold temperatures and the inclusion of substrates during purification. We were therefore surprised that those conditions led to the inactivation of V-ATPase (Moriyama and Nelson, 1989a). Cold inactivation was detected in F-ATPase, but the inactivation could be prevented by including ATP in the incubation medium (Racker, 1976). In contrast, the cold inactivation of V-ATPase depends on Mg-ATP in the medium, and inclusion of EDTA protects against this sensitivity. In addition, treatment by NEM that binds at the ATP binding site of the enzyme has a protective effect as well. Similar effects were observed with yeast vacuoles and membranes containing V-ATPase from other sources including Golgi and lysosomes (Moriyama and Nelson, 1989b,c). Sedimentation of the membranes and analysis of the supernatants of those cold-inactivated preparations revealed that V_1, the catalytic sector, dissociated from the membranes upon the cold treatment. This V_1 has no ATPase activity, and when the cold-treated vacuoles were checked, no H^+ leakage was observed (Beltran and Nelson, 1992). V_1 dissociation was demonstrated in situ in plant and yeast cells as well, by glucose deprivation and during the molting period of insects (Kane, 1995; Sikora *et al.*, 1998; Sumner *et al.*, 1995). Recently we observed that elevated NaCl concentrations in the growth medium of yeast cells have a similar effect to glucose depravation (Perzov *et al.*, 2001). It is obvious that the lack of proton leak by the isolated membrane sector (V_0) and the inactive V_1 are the prerequisites for the development of regulation of V-ATPase by the dissociation and reassociation of its catalytic sector. We assumed that the special properties of V-ATPase stem from the gene duplication of the ancestral gene of the proteolipid and the fact that more than one kind of proteolipid exists in every eukaryotic enzyme (Nelson, 1992; Perzov *et al.*, 2001). Assuming that a rotary mechanism (Junge *et al.*, 1997) also operates in V-ATPase (Nelson *et al.*, 2002), it is sufficient that removal of V_1 blocks the rotation of the proteolipid barrel to stop any proton leak.

The inability of V-ATPase to reach thermodynamic equilibrium was also attributed to the double size of the c subunits and the presence of a single, negatively charged moiety per four transmembrane helices (Nelson

and Nelson, 1989; Perzov *et al.*, 2001). Apparently the key subunit for this property is Vma16p, that was accidentally discovered by Mike Yoffe and his colleagues (Apperson *et al.*, 1990) and was neglected by us for much too long. It appears that this subunit contains five apparent transmembrane helices, but only four of them are embedded in the membrane (Nishi *et al.*, 2003). It has two negatively charged glutamyl residues in the transmembrane parts, but only the one in the third transmembrane helix is necessary for V-ATPase activity (Hirata *et al.*, 1997). This feature rendered the c-ring an asymmetric structure in contrast to the potentially symmetric structure of the corresponding part in F-ATPase. We recently cloned the Vma16 c-DNA from plants (*Arabidopsis thaliana* and lemon fruit) and both complemented the V-ATPase null mutant (*vma16*Δ) in yeast. Sequence analysis revealed that the first helix is missing in the those subunits, yet nevertheless they complement the yeast null mutant (Aviezer-Hagai *et al.*, 2003). Moreover, removal of the first helix from the yeast Vma16p rendered an active subunit (Nishi *et al.*, 2003).

We proposed that the special arrangement of the V-ATPase c-ring subunits is also the key to understanding the inability of the enzyme to reach thermodynamic equilibrium (Nelson *et al.*, 2002). According to this suggestion, at a certain level of proton motive force the bound protons that completed a full cycle in the hydrophobic milieu in the membrane can slip back out. The slip depends on the magnitude of the protonmotive force that, when large enough, drives them toward the cytoplasmic face of the membrane, through the proton entrance pathway in subunit a.

EPILOGUE

A single particle of V-ATPase as seen by electron microscopy revealed that its general structure does resemble indeed that of F-ATPase (Wieczorek *et al.*, 2000; Wilkens *et al.*, 1999). However, a relevant solution for understanding the mechanism of action of the V-ATPase complex is a high-resolution structure. The main question is whether it is feasible at this time for the holoenzyme. A promising source where the V-ATPase is more abundant, is the midgut of *M. sexta* and we hope the invested efforts in this direction will soon bring results. The approach that adopts expression of each subunit separately in *E. Coli*, purification, crystallization, and solving of its structure seemed irrelevant; however, this was done for subunit H (Sagermann *et al.*, 2001) and the solved structure was fitted nicely into the picture of the holoenzyme particle (Grüber *et al.*, 2001). Probably not all the subunits of the V-ATPase complex, especially the hydrophobic ones, could be expressed in this way but the data of subunit H made it clear that it is worthwhile to try and crystallize separately as many subunits as possible.

Arguably, V-ATPase is one of the most fundamental enzymes in nature; why then is it not known to most biology students and many of their teachers? There are several levels at which one can try to explain this phenomenon. One is that it is such a complex enzyme, which transfers the smallest ion from one compartment to the other and this "fuss" is difficult to comprehend by the general public. The second may be that people who research this field are less extravert than others. The third is that the enzyme is vital for almost every eukaryotic cell, and therefore it is unlikely to accumulate mutations that express themselves as human diseases. The latter is most probably the culprit. There is a remedy for the lack of diseases and these are novel membrane complexes that directly interact with V-ATPase subunits or the holoenzyme and they are likely to be susceptible for genetic alterations. One of these is a membrane chaperon family (Vtc proteins), that was discovered in yeast and has no homologues in mammals (Cohen *et al.*, 1999). We believe that analogous proteins exist but the homology was lost during their fast evolution in comparison with the slow evolution of the V-ATPase subunits. Obviously these proteins can tolerate mutations and some of them may lead us to the "Promised Land" of human diseases. For a starter, one may look at possibly existing mutations in the Ac45 subunit of V-ATPase in mammals (Supek *et al.*, 1994a), the homologous subunit of which was never discovered in lower eukaryotes. This will provide us with sufficient publicity and consequent grant support to answer the really interesting question: why eukaryotic V-ATPase fails to reach thermodynamic equilibrium.

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