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THE CELLULAR BIOLOGY OF PROTON-MOTIVE FORCE GENERATION BY V-ATPases

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Summary

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. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form ATP at the expense of the proton-motive force, V-ATPases function exclusively as ATP-dependent proton pumps. The proton-motive force generated by V-ATPases in organelles and across plasma membranes of eukaryotic cells is utilized as a driving force for numerous secondary transport processes. The enzyme is also vital for the proper functioning of endosomes and the Golgi apparatus. In contrast to yeast vacuoles, which maintain an internal pH of approximately 5.5, it is believed that the vacuoles of lemon fruit may have a pH as low as 2. Similarly, some brown and red algae maintain an internal pH as low as 1 in their vacuoles. It was yeast genetics that allowed the identification of the special properties of individual subunits and the discovery of the factors that are involved in V-ATPase biogenesis and assembly.

Null mutations in genes encoding V-ATPase subunits of *Saccharomyces cerevisiae* result in a phenotype that is unable to grow at high pH and is sensitive to high and low metal-ion concentrations. Treatment of these null mutants with ethyl methanesulphonate causes mutations that suppress the V-ATPase null phenotype, and these cells are able to grow at pH 7.5. The suppressor mutants were denoted as *svf* (Suppressor of *V*-ATPase *F*unction). The *svf* mutations are recessive: crossing the *svf* mutants with their corresponding V-ATPase null mutants resulted in diploid strains that were not able to grow at pH 7.5. A novel gene family in which null mutations cause pleiotropic effects on metal-ion resistance or on the sensitivity and distribution of membrane proteins in different targets was discovered. We termed this gene family *VTC* (*V*acuolar *T*ransporter *C*haperon) and discovered four genes in *S. cerevisiae* that belong to the family. Inactivation of one of them, *VTC1*, in the background of V-ATPase null mutations resulted in an *svf* phenotype that was able to grow at pH 7.5. Apparently, Vtc1p is one of a few membrane organizers that determine the relative amounts of different membrane proteins in the various cellular membranes. We utilize the numerous yeast mutants generated in our laboratory to identify the specific organelle whose acidification is vital. The interaction between V-ATPase and the secretory pathway is investigated.

Key words: V-ATPase, vacuolar acidification, chaperon, proton slip, assembly, proton-motive force, yeast, *Saccharomyces cerevisiae*.

Introduction

The secretory pathway is one of the hallmarks of eukaryotic cells. The pathway furnishes the cells with an elaborate membrane network operating in the space between the nucleus and the plasma membrane. It includes all the cellular organelles and membranes, excluding the organelles that emerged as a result of bacterial endosymbiosis (mitochondria, chloroplasts and peroxisomes). The organelles and membranes of the secretory pathway as well as the plasma membrane are connected to each other by a continuous membrane flow that results in exchange of materials and directional sorting of membrane proteins and lipids. Nevertheless, the composition of the membranes of all the organelles connected with the secretory pathway is unique, and the specific internal contents of the organelles are maintained. The assembly and maintenance of this dynamic entity require energy at several levels. Although part of the energy is provided by cytoplasmic metabolic processes, a large part of the energy utilized to build up and maintain the unique composition of the lumen is provided by ion pumps. The vacuolar H^+-ATPase (V-ATPase) provides much of this energy in the form of a proton-motive force. Energetization of organelles by V-ATPase also results in their acidification. The level of acidification is dependent on the extent and quality of secondary transport systems, as well as on the specific properties of the specific V-ATPase operating

in the various organelles. V-ATPase is a highly conserved enzyme, and it is assumed that all family members have a similar, if not identical, mechanism of action (Nelson, 1992; Nelson and Harvey, 1999). A major dilemma arises; how can such a conservative enzyme function in such a wide variety of membranes and physiological processes? In contrast to yeast (*Saccharomyces cerevisiae*) vacuoles, which maintain an internal pH of approximately 5.5, it is believed that the vacuoles of lemon fruit may have a pH as low as 2 (Taiz, 1992; Muller et al., 1996, 1997). Similarly, some brown and red algae maintain a very low pH in their vacuolar system (Ziegler et al., 1995). A function of V-ATPase in stress conditions is now emerging. Particularly striking are those V-ATPases that pump protons into vacuoles with an extremely low pH, approaching 0. This property presents a mechanistic challenge in which the ATP/H^+ stoichiometry may drastically change and/or a proton slip (or change in stoichiometry) may be altered (Moriyama and Nelson, 1988). The blood of the ascidian *Sydneiensis samea* contains giant cells that accumulate rare metals such as vanadium, tantalum and niobium to extremely high concentrations (Michibata, 1993). Recently, the biochemical properties of these cells have been studied, and it was found that V-ATPase comprises approximately 10 % of their total protein (Uyama et al., 1994). The pH inside the vacuole was estimated to be approximately 0.1, representing a proton concentration of approximately $0.8\,mol\,l^{-1}$ (Y. Moriyama, personal communication). The more than 10^7-fold concentration gradient of H^+ from the vacuole to the cytoplasm presents major biological questions including a mechanistic enigma for V-ATPase. Now that the subunit structure of V-ATPases is largely understood, studies on its mechanism of action and the physiology of the systems in which the enzyme is the main energy provider are likely to take centre stage.

Cellular biology of yeast V-ATPase

The yeast *Saccharomyces cerevisiae* has provided an excellent tool for studying the structure, function, biogenesis, assembly and physiology of V-ATPase and the secretory pathway (vacuolar system). Since the V-ATPase provides most of the energy to the vacuolar system and brings about the acidification of its lumen, alterations in the activity of the enzyme result in pleiotropic consequences that change the entire physiology of the system. Several years ago, it was demonstrated that treatment of yeast and mammalian cells with uncouplers results in missorting of proteins in their secretory pathway (Klionsky et al., 1990; Yamashiro et al., 1990). Null mutations in V-ATPase subunits have the same effects (Nelson and Nelson, 1990). Therefore, acidification of the lumen of the secretory pathway is one of the determinants in the correct targeting of membrane and secretory proteins.

So far, only yeast cells have been shown to survive the elimination of V-ATPase activity. Disruption of genes encoding V-ATPase subunits in *Neurospora crassa* or *Drosophila melanogaster* is lethal (Ferea and Bowman, 1996; Bowman et al., 1997; Dow et al., 1997). Very recently, it was reported that disruption of a *VMA1* gene in the fungus *Ashbya gossypii* is not lethal (Forster et al., 1999). It was proposed that yeast V-ATPase null mutants could grow at low pH because they are able to energize their vacuolar system by fluid-phase endocytosis, which provides an acidic medium for the lumen (Nelson and Nelson, 1990). Indeed, inactivation of the genes involved in endocytosis on the background of a V-ATPase null mutation caused lethality (Munn and Riezman, 1994). However, the precise metabolic process that prevents the growth of V-ATPase null mutants at high pH is not known. Moreover, the location of the vital acidic compartment in the vacuolar system is not apparent. Indirect evidence indicates that the vital acidic compartment is not the yeast vacuole (Klionsky et al., 1990). The lack of V-ATPase activity in *S. cerevisiae* resulted in several conditional-lethal phenotypes, including growth arrest at neutral pH, sensitivity to high and low metal-ion concentrations and altered glycosylation pattern and missorting of vacuolar proteins (Stevens and Forgac, 1997; Nelson and Harvey, 1999). Growth at low pH may correct some, but not all, of these defects, presumably by replacing acidification *via* the activity of V-ATPase, by a fluid-phase endocytosis that brings the acidic external fluid into crucial positions in the secretory pathway of the cell (Nelson and Nelson, 1990; Munn and Riezman, 1994). Because of the pleiotropic effect of the V-ATPase null mutations, combinations with some other null mutants are expected to generate a synthetic lethality. Indeed, we have demonstrated that the generation of V-ATPase null mutations in the temperature-sensitive yeast strain *sec7* causes almost complete growth arrest even at the permissive temperature (N. Perzov and N. Nelson, unpublished results). Since Sec7p has been localized to the Golgi complex, this observation may help to identify the organelle whose acidification is vital.

Sporadic growth of V-ATPase null mutants on plates buffered at pH 7.5 was observed, and treatment with ethyl methanesulphonate (EMS) resulted in the generation of these mutants with a frequency of up to 0.1 % (Perzov et al., 1998). The mutants were denoted as *svf* (*S*uppressor of *V*-ATPase *F*unction). The large number of colonies suggests a large target for mutagenesis that may contain multiple proteins. It is likely, but not necessary, that the *svf* phenotype resulted from inactivation of these proteins. Indeed, a diploid strain that had one allele of *svf* and two alleles of a null mutation in one of the V-ATPase subunits was unable to grow on a medium buffered at pH 7.5.

We identified and cloned a yeast gene family containing four genes (*V*acuolar *T*ransporter *C*haperon) *VTC1*, *VTC2*, *VTC3* and *VTC4* (A. Cohen, N. Perzov, H. Nelson and N. Nelson, in preparation). Each of the four genes was disrupted, and the four null strains Δ*VTC1*, Δ*VTC2*, Δ*VTC3* and Δ*VTC4* were studied. Inactivation of *VTC1* resulted in growth at pH 7.5 of V-ATPase null mutants. Therefore, the phenotype of Δ*VTC1* on a background of V-ATPase null mutation is identical to the *svf* mutant. One of the *svf* mutants generated by EMS treatment was complemented by a plasmid bearing *VTC1*.

How can inactivation of a cellular protein suppress the

phenotype of V-ATPase null mutations? One of the possibilities is replacement of V-ATPase in the crucial cellular organelles by another mechanism that generates a proton-motive force. In the absence of active V-ATPase, the best candidate for such rearrangement is the plasma membrane H^+-ATPase (Pma1p). The presence of this P-type ATPase is vital, and it functions in generating a proton-motive force at the plasma membrane as well as in cellular pH homeostasis (Soteropoulos et al., 1998; Mason et al., 1998). Inactivation of the *VTC1* gene resulted in a drastic reduction in the amounts of Pma1p in the plasma membrane (A. Cohen, N. Perzov, H. Nelson and N. Nelson, in preparation). Therefore, we suggest that a small part of the missing Pma1p may find itself in the organelle whose acidification is vital and thus suppresses the lethality of V-ATPase null mutations at pH 7.5. A recent observation that the incidence of *svf* mutations increased tenfold in yeast bearing the null V-ATPase subunit allele together with *sec1-1* supports the above proposal (N. Perzov and N. Nelson, unpublished results). Moreover, mutations of *pma-1*, the gene encoding the plasma membrane H^+-ATPase of *Neurospora crassa*, suppressed inhibition of growth by the V-ATPase inhibitor concanamycin A (Bowman et al., 1997). The identity of the cellular organelle whose acidification is vital is still not known. We know that it is not the yeast vacuole because several yeast mutants that fail to acidify their vacuoles are viable at neutral pH (Klionsky et al., 1990; Banta et al., 1988; Wendland et al., 1988), and the elimination of Vtc1p in the background of V-ATPase null mutations did not result in the acidification of the yeast vacuole (A. Cohen, N. Perzov, H. Nelson and N. Nelson, in preparation). The most likely candidate for the crucial acidifying organelle is one or more of the post-Golgi structures.

Two recent observations indicate that the post-Golgi vesicles are responsible for growth arrest in V-ATPase null mutants. Null mutation in the *GEF1* gene encoding a Cl^- channel resulted in iron requirement and cation sensitivity (Gaxiola et al., 1998). Although the vacuolar acidification was not altered, the yeast grew poorly at high pH, and the localization of the defects suggested post-Golgi structures. Similarly, inactivation of the gene *KEX2* resulted in growth arrest at high pH and a failure of vacuolar acidification (Oluwatosin and Kane, 1998). Further studies revealed that the vacuolar acidification was not altered, and this may be due to reduced amounts of V-ATPase reminiscent of Δ*VTC1* (N. Perzov and N. Nelson, unpublished results). Kex2p is known to be localized at the post-Golgi network. It is suggested that inactivation of these two genes led to an inappropriate proton uptake activity of V-ATPase specifically at the post-Golgi network, resulting in phenotypes having some, but not all, the properties of V-ATPase null mutants.

The *VTC* family members encode chaperons of membrane proteins

The *VTC* family contains four genes in the yeast genome that were denoted as *VTC1*, *VTC2*, *VTC3* and *VTC4* and encode proteins of 14 380 Da, 95 435 Da, 96 551 Da and 75 483 Da, respectively. Vtc1p is a highly hydrophobic protein containing three potential transmembrane helices, whereas Vtc4p appears to be hydrophilic protein with no transmembrane helices. The predicted structure of Vtc2p and Vtc3p appears to be a complex between Vtc1p and Vtc4p (A. Cohen, N. Perzov, H. Nelson and N. Nelson, in preparation). Cell fractionation and immunoblotting with anti Vtc4p revealed that this presumably hydrophilic protein fractionates with the membranes, probably in a complex with Vtc1p. A common new amino acid motif of L*K**XXXX*E*X*Y*XX*L*XX*L*XX*F*XX*LN*X*TGF*X*KI*X*KK*X*DK was identified in the N-terminal hydrophilic part of these proteins, and the motif was shared by several proteins in all phyla containing a general structure consisting of a large hydrophilic N terminus and a membrane-bound C terminus. In *S. cerevisiae*, it includes the proteins Pho81p, Pho87p and Syg1p and the open reading frames YNR013C, YJL198W and YDR089. Potential homologous proteins have been identified in *Caenorhabditis elegans*, *Drosophila melanogaster* and a few expressed sequence tags in mouse and human libraries.

Yeast mutants were generated in which each of the four *VTC* genes was interrupted by the introduction of *URA3*, *LEU2* or *HIS3* to replace most of the reading frames (A. Cohen, N. Perzov, H. Nelson and N. Nelson, in preparation). Except for Δ*VTC1*, which exhibited a reduced or absent *in vivo* vacuolar acidification, the disruptant mutant did not show a V-ATPase-related phenotype. However, isolated vacuoles revealed that the VTC protein influences the distribution of V-ATPase in the vacuole. Fig. 1 shows the ATP-dependent proton uptake activity of vacuoles isolated from the wild type and from the different *VTC* null mutants. In line with the *in vivo* observation, Δ*VTC1* exhibited markedly reduced proton uptake activity in comparison with wild-type vacuoles. Similar low activity was observed for Δ*VTC4* in isolated vacuoles, although no apparent phenotype was found in the *in vivo* vacuolar acidification as measured by quinacrine accumulation. The small differences in the V-ATPase activity between Δ*VTC1* and Δ*VTC4* may result in the detection of quinacrine accumulation in the latter mutant only. The Δ*VTC2* and Δ*VTC3* mutants exhibited higher V-ATPase activity than Δ*VTC1* and Δ*VTC4* in isolated vacuoles. Indirect evidence indicates that the VTC family of proteins affects the distribution of several other membrane proteins, including metal-ion transporters.

When analyzed for growth under metal-ion stress conditions, it appeared that each of the Δ*VTC* mutants exhibited a different growth resistance or sensitivity. Fig. 2 depicts the proposed membrane topography of the various Vtc proteins as well as the sensitivity of the respective deletion mutants to elevated levels of metal ions or to oxidative stress (addition of H_2O_2). In the absence of a recognizable signal sequence, the N termini were placed on the cytoplasmic face of the membrane. The disruptant mutant Δ*VTC1* was resistant to elevated Mn^{2+} concentrations in the medium and more sensitive then the wild type to Zn^{2+}, Cu^{2+} and H_2O_2. The disruptant mutant Δ*VTC4* was also resistant to higher concentrations of Mn^{2+} and sensitive to higher Zn^{2+}

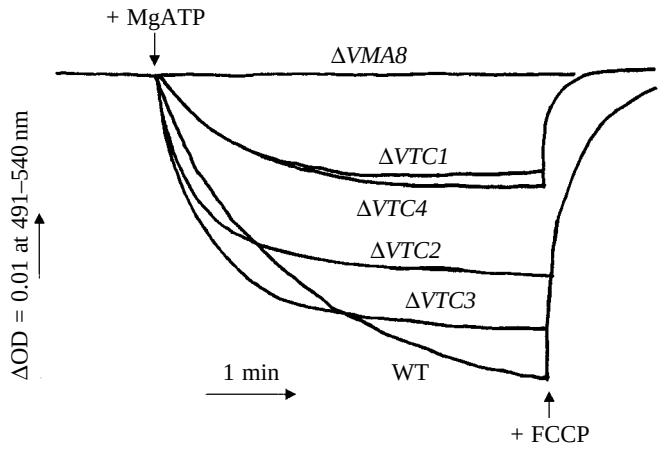

Fig. 1. The effect of *VTC* gene disruption on V-ATPase activity in isolated vacuoles. Yeast vacuoles were isolated as described by Supek et al. (1994) and adjusted to a protein concentration of 5 mg ml^{-1}. That the amounts of protein in the various preparation were equal was verified by comparing the staining intensity of major proteins in SDS–polyacrylamide gels. ATP-dependent proton uptake was measured by following changes in Acridine Orange absorption at 491–540 nm (see Supek et al., 1994). Vacuoles containing 50 µg of protein were assayed in each sample. Where indicated, 10 µl of 0.1 mol l^{-1} MgATP or 1 µl of 1 mmol l^{-1} P-(trifluoromethoxy) phenylhydrazone (FCCP) was added. ΔOD, change in optical density; WT, wild-type; Δ*VMA8*, Δ*VTC1*, Δ*VTC2*, Δ*VTC3* and Δ*VTC4* are the gene disruptant mutants.

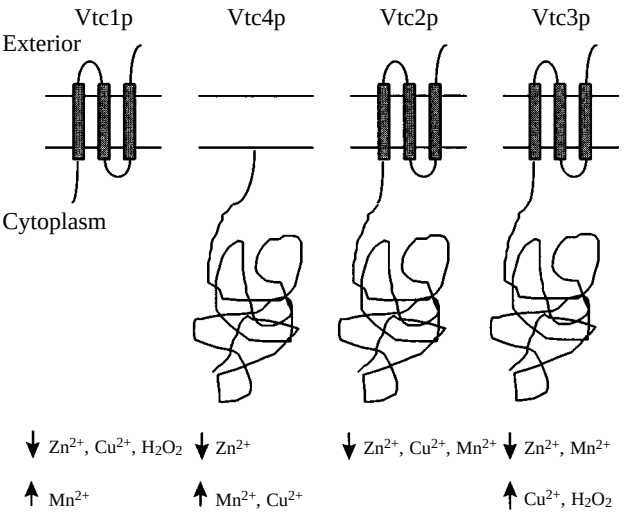

Fig. 2. A proposed membrane structure for the VTC family members (Vtc1p, Vtc2p, Vtc3p and Vtc4p) and the metal-ion sensitivity of their individual null mutants. It is assumed that the hydrophilic N terminus is situated on the cytoplasmic face of the membrane and that the hydrophobic C terminus is anchored to the membrane. Resistance (↑) and sensitivity (↓) to metal ions were analyzed as follows. The wild-type (W303) yeast strain and the gene disruptant mutants Δ*VTC1*, Δ*VTC2*, Δ*VTC3* and Δ*VTC4* were grown in a medium containing 0.5 % yeast extract, 1 % bacto peptone and 2 % dextrose buffered by 50 mmol l^{-1} Mes to pH 5.5 or 6.0. The medium was supplemented with different metal ions as the chloride salt. The initial cell concentration was set to give an optical density of 0.1 units at 600 nm, and growth at 30 °C was followed by measuring the cell density every hour. The following additives were used: 0.5, 1.0 and 1.5 mmol l^{-1} $ZnCl_2$; 0.5, 1.0 and 2.0 mmol l^{-1} $MnCl_2$; 2.0, 4.0 and 6.0 mmol l^{-1} $CuCl_2$; 0.5 and 2.0 mmol l^{-1} H_2O_2. The experiment was performed in triplicate and repeated at least three times. Sensitivity or resistance was determined by at least 30 % growth inhibition or promotion in comparison with the growth of the wild-type strain under identical conditions.

concentrations but in contrast to Δ*VTC1*, it was also resistant to elevated Cu^{2+} concentrations in the medium. Unlike Δ*VTC1*, Δ*VTC4* was not sensitive to H_2O_2. Δ*VTC2* exhibited sensitivity to Zn^{2+}, Cu^{2+} or Mn^{2+} and no resistance to elevated metal-ion concentrations. Δ*VTC3* showed sensitivity to Zn^{2+} or Mn^{2+} and resistance to Cu^{2+} or H_2O_2.

The influence of Vtc1p on membrane protein distribution can be attributed to its involvement in the synthesis, biogenesis, trafficking or degradation of the affected membrane proteins. We propose that VTC proteins are involved in a novel biological function that could be defined as 'minding the inventory'. The question of what determines the amounts of various membrane proteins in the different organelles and membranes is frequently attributed to the balance between their synthesis and degradation. We think that this is only part of the mechanism and that it has to be complemented by a system that checks the inventory and reports it to the synthetic and degradation pathways. We propose that the VTC family of proteins fulfils this function of 'minding the inventory'.

Subunit structure and mechanism of action

One of the most intriguing questions concerning proton pumps is the number of subunits required to perform the simple enzymatic reaction of ATP-dependent proton uptake. In contrast to the plasma membrane proton pump Pma1p, which is mainly composed of a single polypeptide, V-ATPase is composed of at least 11 subunits and its molecular mass approaches 10^6 Da (Nelson and Harvey, 1999). Why are there so many subunits and how was the enzyme formed in the dawn of life? The primordial V-ATPase (F-ATPase presumably evolved from this primordial V-ATPase) is thought to have been composed of hexamer of a single protein (Nelson, 1992). The evolutionary driving force to add more and more subunits may have been the selection for a higher efficiency of catalysis by a single molecule. Because of the statistical element in biochemical reactions, there is no great difference between the reactions catalyzed by small units compared with very large units. This rule is drastically changed when the reaction is catalyzed by a single molecule (Mehta et al., 1999; Qian and Elson, 1999). In this case, fluctuation becomes an overwhelming element in the catalytic activity of small molecules (Dickson et al., 1997; Moerner and Orrit, 1999). This 'blinking' or 'breathing' is reminiscent of a single-channel conductance (Nelson et al., 1980). We propose that F- and V-ATPases evolved as multi-subunit enzymes to depress these fluctuations and consequently evolved into molecular machines. The increase in size reduced the differences between

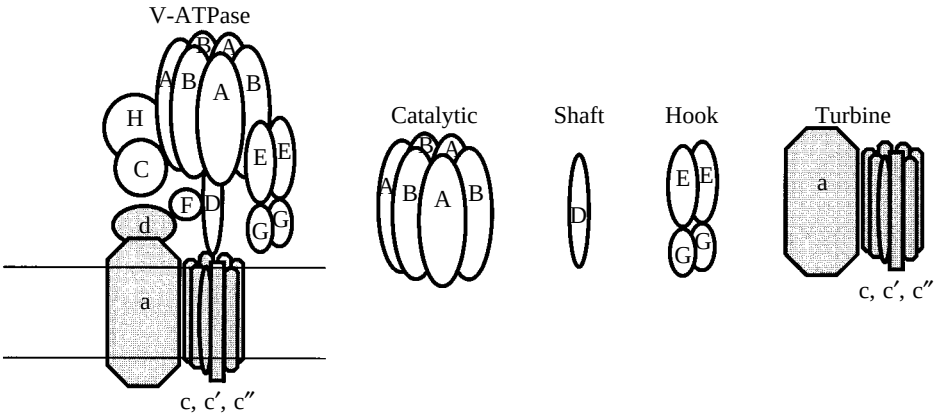

Fig. 3. Subunit structure of V-ATPase. The proposed organization of the various subunits is based on studies described in the text and assuming a common general structure between F- and V-ATPases. The subunit nomenclature is according to Stevens and Forgac (1997) (see also Nelson and Harvey, 1999). The yeast genes encoding the listed subunits are: A, *VMA1*; B, *VMA2*; C, *VMA5*; D, *VMA8*; E, *VMA4*; F, *VMA7*; G, *VMA10*; H, *VMA13*; a, *VPH1* and *STV1*; c, *VMA3*; c′, *VMA11*; c″, *VMA16*; d, *VMA6*.

the catalytic activity of single and multiple enzymes. This uniformity enables the system to convert the chemical energy of ATP hydrolysis into a torque of precisely 120 ° turn for each ATP molecule hydrolysed (Yasuda et al., 1998). Similar events may have occurred during the evolution of other molecular machines, such as the flagellar motor and DNA and RNA polymerases (DeRosier, 1998; Gelles and Landick, 1998; Makiniemi et al., 1999).

The general structures of F- and V-ATPases are quite similar. Both holoenzymes are composed of catalytic sectors, F_1 and V_1, respectively, and membrane sectors, F_o and V_o, respectively. The mechanism of action of F-ATPase has been described as a molecular motor (Boyer, 1993; Abrahams et al., 1994; Junge et al., 1997). Accordingly, we assigned the V-ATPase subunits to four parts of a mechanochemical machine (Nelson and Harvey, 1999): (i) a catalytic unit, (ii) a shaft, (iii) a hook and (iv) a turbine. Subunits A and B of the catalytic sector are thought to function in a similar, if not identical, fashion to F-ATPase subunits β and α, respectively. Less confidently, subunits E and G (Vma4p and Vma10p) are thought to function in the 'hook' and the proteolipids (subunits c) in the proton 'turbine'. Subunit D (Vma8p) is the best candidate for the rotating 'shaft' (analogous to subunit γ of F-ATPase). Fig. 3 depicts the proposed subunit structure and function of V-ATPase.

One of the intriguing questions that remain unanswered, not only for V-ATPase but also for most of the other multisubunit protein complexes, is how the nonhomologous subunits are related to each other and how they evolved. V-ATPase and the other multisubunit protein complexes belong to the current 'protein world' that came after the prebiotic worlds such as the iron–sulphur and RNA worlds (see Baltscheffsky, 1996; Cammack, 1996; Cech, 1993; Patel, 1998; Levy and Miller, 1999). To understand the evolution of V-ATPase, we have to suggest logical events that led to the current enzyme and to answer some very difficult questions. What were the properties of the ancestral enzyme and what functions did it have initially? Which functions arose later in evolution? We have no alternative but to look for clues to the answers to these questions in extant organisms from different phyla and to extrapolate backwards as far as possible. The evolution of the homologous subunits in F- and V-ATPases has been discussed in several papers, and evolutionary trees have been constructed for each of these subunits (Nelson, 1992; Gogarten et al., 1992; Taiz and Nelson, 1996).

The presence of V-ATPase in all phyla including the eubacteria and the presence of F-ATPase in eubacteria and eukaryotes suggest a free evolutionary competition between them regarding the function of the universal proton pump and/or ATP synthase. The advantage of F-ATPase stems from its precision and high degree of coupling. In contrast, the advantage of V-ATPase may stem from its functional flexibility and its variable degree of coupling, resulting in a controlled slip (Moriyama and Nelson, 1988). The molecular mechanism that distinguishes the two enzymes is not known. Even though there are significant differences in the size, amino acid sequences and evolutionary origin of the catalytic sectors of the two enzymes, there is no indication that the 'slip' control can be attributed to them. However, the subunit structure of the V-ATPase membrane sector suggests that most of the unique properties of the enzyme evolved through changes in the genes encoding subunits of the membrane sector or 'turbine' (Nelson, 1992; Nelson and Harvey, 1999). Duplication and fusion of the gene encoding the eukaryotic subunit c (proteolipid) of V-ATPase was the most important factor in the evolution of the enzyme into an exclusive proton pump controlled by a slip (Mandel et al., 1988; Nelson and Nelson, 1989, 1990; Umemoto et al., 1990; Nelson, 1992). The discovery of two additional proteolipids c′ and c″ in eukaryotic V-ATPases, each of which also evolved by gene duplication and fusion of an ancestral proteolipid, suggests that they too may be involved in conferring the unique properties of the enzyme (Apperson et al., 1990; Umemoto et al., 1991; Hirata et al., 1997). Why the lemon is sour is much more than a popular scientific question (Muller et al., 1996, 1997). Solving the molecular mechanism of this process may elevate our understanding of one of the most fundamental bioenergetic processes to a new much higher level.

The physiological virtue of 'slip'

The degree of coupling of biological processes encompasses

a major theme in several theoretical and experimental studies (Van Dam, 1994; Westerhoff et al., 1995). Both disciplines indicated that in almost every biological process 100 % coupling is unfavourable and is essentially deleterious to living organisms. In energy-producing organelles such as mitochondria and chloroplasts, the respective processes of respiration and photosynthesis are maintained at a relatively high degree of coupling. However, the energy-consuming organelles may utilize coupling modulation as part of the regulation of their bioenergetic processes. The apparent variable stoichiometry of proton-to-ATP coupling in V-ATPases has led to the suggestion that V-ATPase is a proton pump controlled by a 'slip' (Moriyama, and Nelson, 1988). The physiological significance of this mechanism is explained in terms of prevention of overacidification in internal organelles such as synaptic vesicles and chromaffin granules. It is logical to assume that, in those organelles (such as lemon vacuoles) in which internal pH is maintained at pH 2 or below, most of the slip was cancelled and the degree of coupling was elevated to the optimal level of approximately 90 %. Therefore, a molecular solution of the proton slip in the mechanism of V-ATPase may elucidate a fundamental life property of utilizing the imperfect as a virtue. In the course of our studies on other transport processes, we came to realize that there is no biological process without a slip and that understanding transport processes requires the mechanism of their slippage to be unravelled.

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