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MINI-REVIEW

Structure, Molecular Genetics, and Evolution of Vacuolar H^+-ATPases

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Abstract

Proton-ATPases can be divided into three classes denoted as P-, F-, and V-ATPases. The P-ATPases are evolutionarily distinct from the F- and V-type ATPases which have been shown to be related, probably evolved from a common ancestral enzyme. Like F-ATPases, V-ATPases are composed of two distinct structures: a catalytic sector that is hydrophilic in nature and a hydrophobic membrane sector which functions in proton conduction. Recent studies on the molecular biology of vacuolar H^+-ATPases revealed surprising findings about the evolution of pronon pumps as well as important clues for the evolution of eukaryotic cells.

Key Words: Proton-ATPase; vacuolar system; molecular biology; evolution, structure and function; eukaryotic cells; transport.

Introduction

The eukaryotic cell is defined by a separate nucleous 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 vacuolar system contains several organelles that are vital for the reproduction and metabolism of the cell. These organelles maintain an interior composition different from that of the cytoplasm. Concentration gradients of some soluble materials are maintained by continuous pumping across the membranes. Several organelles maintain an interior pH lower than the cytoplasm by pumping protons into the organelles via an ATP-dependent process. The enzyme which catalyzes

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this reaction is denoted as the vacuolar H^+-ATPase or V-ATPase (Mellman *et al.*, 1986; Al-Awqati, 1986; Bowman *et al.*, 1988c). The protonmotive force generated by this enzyme is utilized for several energy-dependent processes, and the internal acidic pH is vital for numerous biochemical reactions which take place inside the organelles. Organelles which contain the vacuolar H^+-ATPase are synaptic vesicles (Cidon and Shira, 1989; Moriyama and Nelson, 1989b), chromaffin granules (Percy and Apps, 1986; Cidon and Nelson, 1986), platelet-dense granules (Rudnick, 1986), several secretory granules such as parafollicular granules (Barasch *et al.*, 1988), plant and fungal vacuoles (Uchida *et al.*, 1985, 1988; Bennett *et al.*, 1984), lysosomes (Moriyama and Nelson, 1989b), clathrin-coated vesicles (Xie and Stone, 1986; Arai *et al.*, 1987a, b), the Golgi complex (Chanson and Taiz, 1985; Young *et al.*, 1988), and probably several other organelles. Degradative processes are dependent on the acidic pH inside the organelles, as well as conversion of proinsulin to insulin (Rhodes *et al.*, 1987) and metabolism of catecholamines (Njus *et al.*, 1986). Therefore, the activity of V-ATPases is probably vital for every eukaryotic cell.

Proton-ATPases can be divided into three main classes: (a) plasma membrane-type, which operates via phosphoenzyme intermediate and therefore is part of the superfamily of P-ATPases (Bowman and Bowman, 1986; Serrano *et al.*, 1986; Pedersen and Carafoli, 1987; Nelson, 1988); (b) the eubacterial-type F-ATPase enzymes that are present in eubacteria, mitochondria, and chloroplasts; (c) the vacuolar-type V-ATPases, present in archaebacteria and the vacuolar system of eukaryotic cells. While V and F H^+-ATPases are structurally and functionally related, they are distinguished in eukaryotic cells by the distribution of their genes. The genes encoding all the subunits of V-ATPases are present in nucleus, while the genetic information for the F-ATPases is shared by the nuclear and organellar DNA. It is the purpose of this review to discuss the structural relations between these two families of proton-ATPases and to reveal new insight into the evolution of these enzymes.

Structure

Different preparations of V-ATPases were reported to contain various amounts of polypeptides. The purified enzymes from plant and fungal sources were shown to contain three subunits of about 70, 60, and 17 kDa (Uchida *et al.*, 1985; Mandala and Taiz, 1986; Randall and Sze, 1986). On the other hand, preparation of V-ATPases from mammalian sources contained up to 13 different polypeptides (Gluck and Caldwell, 1987). Early studies indicated that the common denominator of all the V-ATPases is the presence of the

above three polypeptides, the 70-kDa subunit contains the ATP and NEM binding sites, and the 17-kDa polypeptide binds DCCD (Bowman and Bowman, 1986). In the last year it became apparent that V-ATPases like F-ATPases are composed of two distinct sectors: a catalytic sector that can be liberated from the membrane by a variety of mild treatments and a membrane sector composed of hydrophobic polypeptides. Winkler and his colleagues were the first to observe particles on chromaffin granules attributed as their proton pumps (Winkler *et al.*, 1986). Recently it became apparent that the basic subunit structure of these enzymes may be quite uniform. The catalytic sector is composed of five subunits and the membrane sector contains two highly hydrophobic polypeptides (Moriyama and Nelson, 1989a, b). Additional accessory subunits were identified in V-ATPases from mammalian sources.

Subunit Structure of the V-ATPases from Bovine Chromaffin Granules. A large body of evidence has clearly shown that an H^+-ATPase is responsible for generating the protonmotive force which is utilized for the uptake of cateocholamines into chromaffin granules (Njus *et al.*, 1986). The identity of this enzyme was revealed by treatment of the membranes with sodium bromide, which inactivated the contaminating mitochondrial enzyme (Cidon and Nelson, 1983). Under these conditions, the chromaffin granule's enzyme was not affected. Subsequently, the chromaffin granule enzyme was purified and reconstituted into liposomes to give ATP-dependent proton uptake (Cidon and Nelson, 1986; Percy and Apps, 1986; Moriyama and Nelson, 1987a). Initially, five major polypeptides were identified in the preparation, neglecting four minor ones that were apparent in the stained gels but were present at lower stoichiometry than the others (Moriyama and Nelson, 1987a). However, the presence of those polypeptides was noticed in preparations of V-ATPase from clathrin-coated vesicles and kidney microsomes (Xie and Stone, 1986; Arai *et al.*, 1987a, b; Gluck and Caldwell, 1987). Cold inactivation studies revealed that all those polypeptides are subunits of the enzyme from chromaffin granules (Moriyama and Nelson, 1989a).

Incubation of membranes containing V-ATPase on ice resulted in inactivation of proton-pumping and ATPase activities of the enzyme (Moriyama and Nelson, 1989a, b). Inactivation is dependent on the presence of anions and MgATP. The cold inactivation resulted in the release of five polypeptides from the membrane with apparent molecular masses of 72, 57, 41, 34 and 33 kDa on sodium dodecyl sulfate gels. Four of the polypeptides of 72, 57, 34 and 33 kDa were identified as subunits of the V-ATPase by antibody cross-reactivity and by amino acid sequencing. Using cold inactivation, similar subunit structure has been revealed for V-ATPases from every source tested so far, including plant vacuoles. Therefore, it appears that the catalytic sector of V-ATPases is released from the membrane by cold

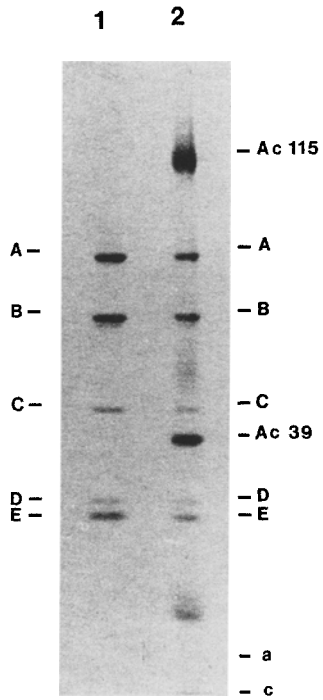

Fig. 1. Subunit structure of the purified V-ATPase from chromaffin granules and the polypeptides released by cold inactivation of the enzyme. Lane 1, reconstituted enzyme was incubated at 0°C for 1 h in the presence of 200 mM NaCl and 5 mM MgATP. Following centrifugation at 200,000 *g* for 30 min, the supernatant was concentrated fivefold and a sample containing about 50 μg protein was electrophoresed on 10% acrylamide gel in the presence of SDS. Lane 2, the purified enzyme (about 50 μg protein) was electrophoresed on 10% acrylamide gel in the presence of SDS. The gel was stained by Coomassie blue. Subunits *a* and *c* stained poorly by this staining procedure. Ac115 and Ac39 are the accessory polypeptides with apparent molecular masses of 115 and 39 kDa, respectively.

inactivation, and the basic subunit structure of this sector contains five different polypeptides denoted as subunits *A* to *E* in the order of decreasing molecular weights from 72 to 33 kDa (Fig. 1). Following cold inactivation of the reconstitued V-ATPase from chromaffin granules, the pellet of high-speed centrifugation contains four major polypeptides. Their apparent molecular weights on SDS gels are 115, 39, 20 and 16 kDa. The 20 (subunit *a*) and 16 (subunit *c*) kDa polypeptides are part of the membrane sector the enzyme, and the polypeptides denoted as Ac115 and Ac39 serve as accessory subunits (Wang *et al.*, 1988). Figure 2 depicts a proposed model for the subunit structure of A-ATPase from chromaffin granules. A similar model has been proposed for the enzyme from clathrin-coated vesicles in which the stoichiometry of the various subunits was precisely determined (Arai *et al.*,

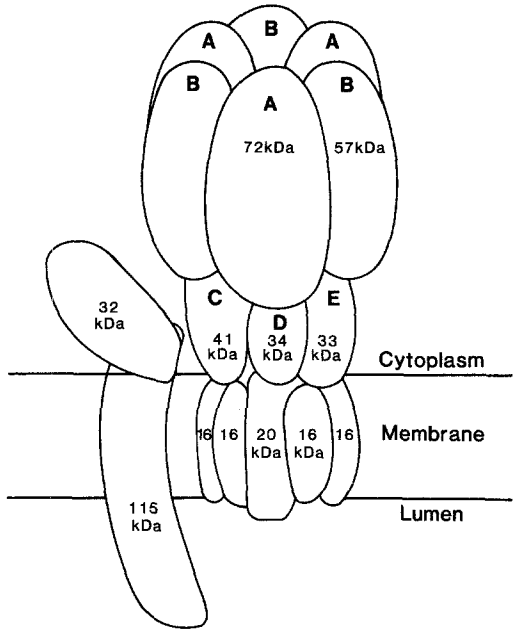

Fig. 2. A model for the subunit structure of V-ATPase from chromaffin granules. The Ac39 subunit is marked as 32 kDa according to its calculated molecular weight (Wang *et al.*, 1988). Six copies of the 16-kDa proteolipid and a few copies of 32- and 115-kDa subunits per enzyme are present.

1988). Using the same method of electroblotting of the purified V-ATPase from chromaffin granules, followed by quantitative amino acid analysis, a stoichiometry of 3 : 3 : 1 for subunits *A*, *B*, and *C*, respectively, was obtained (Moriyama, Miedel, and Nelson, unpublished). The general structure of V-ATPase from plant vacuoles and mammalian clathrin-coated vesicles has been studied using chaotropic agents, reconstitution, and chemical modification (Rea *et al.*, 1987; Lai *et al.*, 1988; Arai *et al.*, 1988; Xie and Stone, 1988). A general structure of peripheral and membrane sectors is widely accepted. Table I summarizes the subunit structure of some of the more defined preparations of V-ATPases from various sources and recent results obtained by analyzing the polypeptides released by cold inactivation of membranes containing the enzyme. A basic subunit structure of five polypeptides in the catalytic sector and at least two polypeptides in the membrane sector was revealed. We predict that this basic structure will hold even for those enzymes from plant and fungal sources that were reported to contain only three different polypeptides.

The precise function of each subunit of the vacuolar enzymes is not clear. The two major subunits (*A* and *B*) of the catalytic sector are the best

Table I. Subunit Structures of Eubacterial and Vacuolar H^+-ATPases[a]

	Catalytic					Membrane				
	A	*B*	*C*	*D*	*E*	*a*		*c*	Accessory	
V-ATPase										
Mammalian										
chromaffin granules	72	57	41	34	33	20		16	115	39
synaptic vesicles										
clathrin-coated vesicles										
lysosomes	72	57	41	34	33	18		15		39
kidney microsome										
Plants	69	55	44		33			17		
Fungi	67	57						16		
Archaebacteria	64	54	28					9		
F-ATPase	α	β	γ	δ	ε	a	b	c		
E. coli	55	50	31	19	15	30	17	8	14	
Chloroplasts	55	54	36	20	15	27	17 +14	8		
Mitochondria	55	52	30	20 (OSCP)	15 (δ) 6 (ε)	30	20	8	About 5 polypeptides	

[a]Molecular weight values in kilodaltons are taken from references cited in the text.

characterized. The A subunit binds the nucleotide analog NBD-Cl and the sulfhydryl reagent NEM in an ATP-protectable fashion (Mandala and Taiz, 1986; Percy and Apps, 1986; Bowman *et al.*, 1986; Moriyama and Nelson, 1987b). Subunit *A* also binds ATP and other nucleotides, and the effect of NEM treatment on the nucleotide binding strongly suggests that this is the catalytic subunit of the enzyme. Therefore, subunit *A* of V-ATPases is homologous to the β subunit of F-ATPases. It is worth noting that treatment with NEM at a concentration that allows exclusive interaction with subunit *A* totally prevented the release of the catalytic sector from the membrane by cold inactivation (Moriyama and Nelson, 1989a). This suggests that the MgATP should bind onto the catalytic site in order to facilitate the cold inactivation of the enzyme. The *B* subunit binds the nucleotide analog BzATP, but with kinetics consistent with a regulatory binding site (Manolson *et al.*, 1985). This is consistent with the assumption that the *B* subunit of V-ATPases is homologous to the α subunit of F-ATPases. The role of the other subunits of the catalytic sector is not known. The amino acid sequence of subunit *E* revealed no homology with any subunit of F-ATPases (Hirsch *et al.*, 1988; Moriyama *et al.*, unpublished).

The proteolipid of V-ATPases is the most extensively studied subunit of these enzymes. This polypeptide (subunit *c*) of about 16 kDa binds DCCD and is soluble in chloroform/methanol. It was first isolated from a chloroform/methanol extract of chromaffin granule membranes (Sutton and Apps, 1981). The vacuolar proteolipid has been isolated and studied from a

wide variety of membranes, including brain clathrin-coated vesicles and plant vacuoles (Arai *et al.*, 1987a, b; Sun *et al.*, 1987; Bowman, 1983; Uchida *et al.*, 1985; Manolson *et al.*, 1985; Randall and Sze, 1986). Binding of DCCD to the protolipid inhibits both the proton pumping and ATPase activities of V-ATPases. The isolate proteolipid was reported to induce a DCCD-sensitive proton conduction in reconstituted phospholipid vesicles (Sun *et al.*, 1987). However, the data were not conclusive enough to eliminate a possible presence of the 20-kDa polypeptide in the preparation. The vacuolar proteolipid is homologous to the proteolipid of F-ATPases (Hoppe and Sebald, 1984; Futai *et al.*, 1988, 1989; Mandel *et al.*, 1988), and in analogy to the membrane sector of F-ATPases, it may require at least one additional subunit for conducting protons across the membrane (Schneider and Altendorf, 1987). The 20-kDa hydrophobic polypeptide of the V-ATPases is a good candidate for this function, and it was denoted as the *a* subunit in analogy to the *a* subunit of the membrane sector of F-ATPases (Moriyama and Nelson, 1989b).

The function of the accessory subunits is not known. So far, they were identified only in V-ATPases from mammalian sources. Although several polypeptides may fall into this category, only two, with molecular masses of about 115 and 39 kDa, consistently copurified with the enzyme. The Ac39 polypeptide is present in all the mammalian enzymes. The gene encoding the polypeptide of chromaffin granules was cloned and sequenced (Wang *et al.*, 1988). Northern hybridization revealed its presence in a wide variety of tissues, and large amounts of the mRNA for this protein were found in cells enriched with lysosomes. No information about the function and origin of this subunit could be generated. The Ac115 polypeptide is a glycoprotein and therefore should face the internal side of the granule (Moriyama and Nelson, unpublished). This polypeptide was identified in enzyme preparations from chromaffin granules, synaptic vesicles, and clathrin-coated vesicles (Xie and Stone, 1986; Cidon and Nelson, 1986; Arai *et al.*, 1987a, b). Studies involving specific antibody against this polypeptide revealed that it is absent from lysosomes, kidney microsomes, and Golgi complex (Moriyama and Nelson, 1988a, 1989b). It is worth noting that the Ac115 polypeptide of the V-ATPases should not be confused with the vanadate-sensitive ATPase of the same molecular weight, which is also present in chromaffin granules, synaptic vesicles, and clathrin-coated vesicles, but is not a proton pump (Moriyama and Nelson, 1988a). Reconstitution studies of the enzyme from clathrin-coated vesicles suggest that the Ac115 polypeptide confers Mg^{++}-ATPase activity to the enzyme (Xie and Stone, 1988). Since this subunit is present only in those organelles that fuse with the plasma membrane, it is tempting to suggest that the 115-kDa polypeptide of V-ATPases is involved in the retrieval of these organelles from the membrane. Therefore, studies

on the biogenesis of V-ATPases may shed light on the function of the accessory polypeptides.

Molecular Genetics of V-ATPases

It was only in the last year that genes encoding subunits of V-ATPases were cloned and sequenced. The genes encoding subunits *A* and/or *B* of the catalytic sector of the enzymes from carrot (Zimniak *et al.*, 1988), *Arabidopsis* (Manolson *et al.*, 1988), *Neurospore* (Bowman *et al.*, 1988a, b), and yeast (Nelson *et al.*, 1989) vacuoles were sequenced. Subunit *c* (proteolipid) of the membrane sector of the enzyme from bovine chromaffin granules and yeast vacuoles was also sequenced (Mandel *et al.*, 1988; Nelson and Nelson, 1989). The sequence information revealed extensive homology among the various subunits of V-ATPases from different sources. At the same time, two of the subunits of the ATP-synthase of the archaebacterium *Sulfolobus acidocaldarius* were cloned and sequenced (Denda *et al.*, 1988a, b). Recently subunits *A* and *B* of *Methanosarcina barkeri* ATPase were also sequenced (Inatomi *et al.*, 1989). It was of no surprise to some of us that the archaebacterial H^+-ATPase resembles the eukaryotic V-ATPase more than it does the eubacterial F-ATPase (Nanba and Mukohata, 1987; Nelson, 1988). Some biochemical features, such as inhibitor sensitivity and the size of some of the subunits, suggested close relations among the archaebacterial ATP-synthase and the V-ATPases (Nelson, 1988). The surprising revelation was the extent of identity among these enzymes. Therefore, it is more appropriate to use the same nomenclature for the archaebacterial subunits as is used for the V-ATPases (see Table I). Recent structural studies of the enzymes from *Sulfolobus* and *Halobacterium* revealed a close relation in the shape of the archaebacterial enzymes to that of F-ATPases (Lubben and Schafer, 1987; Lubben *et al.*, 1987; Nanba and Mukohata, 1987; Mukohata *et al.*, 1987; Mayer *et al.*, 1987). It was shown that subunits *A* and *B* of the enzyme from *Sulfolobus* alternate in the same way that the α and β subunits of F-ATPases do (Lubben *et al.*, 1987). Therefore, the archaebacterial enzyme, which is a V-ATPase type according to the sequence information, has a structure similar to the other ATP-synthases of the F-ATPase type. However, these studies fail to reveal the exact number of the subunits comprising the enzymes from archaebacteria and the relationship between them, and the γ, δ, and ε subunits of F-ATPases are not known. Further sequence information of the equivalent subunits of archaebacteria is vital for our knowledge about the precise relations of these subunits.

Table II shows the percent sequence identity among the various H^+-ATPases. Subunits *A* and *B* are highly conserved and the *A* subunits maintain

Table II. Perrentage of Identical Amino Acids among α and β Subunits of F-ATPases and *A* and *B* Subunits of V-ATPases

V-ATPases	Percent identity
A Neurospora–A carrot	62
A Neurospora–A Sulfolobus	50
B Arabidopsis–B Neurospora	76
B Arabidopsis–B Sulfolobus	53
V versus F-ATPases	
A to *B*	22–26
A to α	18–23
A to β	23–30
B to α	24–28
B to β	24–28
α to β	22–26

about 60% identity between higher plants and fungi. The *B* subunit is even more extensively conserved, it maintained over 70% identity between higher plants and fungi. Remarkably, the sequence identity among the respective subunits *A* and *B* from archaebacteria and the vacuolar enzymes from eukaryotic cells is about 50%. Homologies among the subunits of F-ATPases and V-ATPases were also detected. While subunit *A* of the vacuolar enzymes showed better homology with β subunits of F-ATPases, the *B* subunits had a closer relation to the α subunits of the eubacterial enzymes (Nelson and Taiz, 1989). The same relations hold for the enzymes from archaebacteria and eubacteria, demonstrating that even though the archaebacterial H^+-ATPase is not merely a proton pump but also functions as ATP-synthase, it is closely related to the vacuolar enzymes, which can no longer synthesize ATP (Nelson and Nelson, 1989).

The concensus sequences for the ATP-binding site was detected on the *A* subunit of enzymes from the vacuolar system of eukaryotic cells and the archaebacterial ATP-synthase (Walker *et al.*, 1982; Taylor and Green, 1989; Zimniak *et al.*, 1988; Denda *et al.*, 1988a). Subunit *A* of the enzyme from carrot contains five cysteine residues, three of which are located in the putative active site region (Zimniak *et al.*, 1988). The presence of these cysteine residues in the vacuolar enzymes and their absence from the F-ATPase may explain the sensitivity of the former enzymes to NEM (Bowman and Bowman, 1986). NBD-Cl inhibits both F-ATPases and V-ATPases (Nelson, 1988). The inhibition of F-ATPases by this reagent is caused by binding to a single tyrosine residue on the β subunits of these enzymes. This tyrosine residue is located in a conserved region of both the β subunits of F-ATPases and the *A* subunit of V-ATPases. However, the corresponding tyrosine is missing in the vacuolar enzymes, where the tyrosine was replaced by serine (Zimniak *et al.*, 1988). It may be that NBD-Cl inhibits the V-ATPases by

binding to serine or to one of the cysteine residues located in the active site of the enzyme (Moriyama and Nelson, 1987b). Bound ATP is readily cross-linked to the active site of the enzyme from chromaffin granules by UV illumination (Moriyama and Nelson 1987b). On the other hand, similar treatment gives very poor results with F-ATPases (unpublished). The best candidate for cross-linking ATP is a tryptophan residue at or near the ATP-binding site of the enzyme. Tryptophan is missing from all the β subunits of the F-ATPases sequenced so far. The two *A* subunits of V-ATPases that have been sequenced contain a few tryptophan residues; five of them were conserved in the *A* subunits from carrot and *Neurospora* (Zimniak *et al.*, 1988; Bowman *et al.*, 1988a). Among them the conserved tryptophan residues at position 357 of carrot and 351 of *Neurospora* flank the second part of the proposed universal ATP-binding site (Taylor and Green, 1989). This tryptophan may be involved in the photochemical binding of ATP to the *A* subunit. A comprehensive survey of all the mutants of the β subunit of F-ATPase from *E. coli* and comparison of the corresponding amino acids in the *A* subunit of V-ATPase from *Neurospora* revealed that about 50% of them were conserved in the V-ATPase (Bowman *et al.*, 1988a). The comparison may stress the value of site-directed mutagenesis, but it also pointed out the weakness of the method in reaching sound mechanistic conclusions.

The genes encoding subunits *B* of V-ATPases from various sources were cloned and sequenced (Bowman *et al.*, 1988b; Manolson *et al.*, 1988; Nelson *et al.*, 1989). The deduced amino acid sequences showed remarkable conservation with about 80% identity between the polypeptides from plant and fungal sources. By removing from consideration the N- and C-termini, the identity is greater than 90%. The *B* subunit of V-ATPases shows significant homology to both the α and β subunits of F-ATPases. Although the overall homology is about 25%, there are two stretches of amino acid sequences with about 50% identity between the corresponding sequences of *B* subunit of V-ATPases and α and β subunits of F-ATPases (Nelson and Nelson, 1989). The significance of these homologies will be discussed in the section on evolution.

The gene encoding subunit *E* from V-ATPase of kidney microsomes was cloned and sequenced (Hirsch *et al.*, 1988). It showed no homology with sequences of F-ATPase subunits. It has a short region with limited homology to P-ATPases. The positive identity of this *c*DNA as the gene encoding subunit *E* can from amino acid sequences obtained for the isolated subunit of the enzyme from chromaffin granules (Moriyama *et al.*, unpublished). The amino acid sequences obtained for the isolated subunit *E* were identical with the corresponding sequences deduced from the *c*DNA of the kidney enzyme. Since the two tissues are derived from different embryonic origin the finding suggests that a similar if not identical enzyme operates in all the vacuolar

organelles, and the organellar specificity may come from accessory polypeptides (Moriyama and Nelson, 1989b). The *c*DNA encoding such an accessory polypeptide was cloned and sequenced, but the sequence provides no revelation for the mode of action of this subunit (Wang *et al.*, 1988).

The membrane sector of V-ATPases contain at least two hydrophobic polypeptides denoted as subunits *a* and *c* (Forgac, 1989; Moriyama and Nelson, 1989b). The *c*DNA encoding the *c* subunit (proteolipid) of the enzyme from chromaffin granules was cloned and sequenced (Mandel *et al.*, 1988). The gene encodes a highly hydrophobic protein of about 16 kDa. Hydropathy analysis revealed four trans-membrane segments, the fourth of which contains a glutamic acid residue that is likely to be the DCCD binding site. All of the sequences of proteolipids of F-ATPases, which contain glutamic acid as the binding site for DCCD, aligned perfectly with the corresponding glutamic acid of the V-ATPase. Recently, the gene encoding the proteolipid of yeast V-ATPase was cloned and sequenced (Nelson and Nelson, 1989). The amino acid sequence of the fourth trans-membrane segment was 100% identical with that of the proteolipid from chromaffin granules. This is a unique example of tight conservation of hydrophobic stretch of amino acids because seveal hydrophobic amino acids are considered to be mutually replaceable. In the case of the proteolipids from V-ATPases, the most conserved parts are the trans-membrane segments. The sequences of proteolipids from mammalian and fungal sources provided additional evidence for the strict conservation of the subunits of V-ATPases from the different organelles and various eukaryotic cells. This notion is supported by the observation that in yeast cells a single gene encodes subunits *B* and *c* (proteolipid) of the V-ATPase (Nelson *et al.*, 1989; Nelson and Nelson, 1989). Unlike the F-ATPase that is located exclusively in mitochondria, V-ATPase are present in several organelles with different functions. So far, N-Terminal signal sequences were not detected in genes encoding subunits of V-ATPases. This raises the question of the way by which the enzymes are segregated to the various organelles. There are several possibilities to fulfill this function, including post-translational modification for targeting the subunits and involvement of accessory polypeptides that are organelle specific.

Evolution of H^+-ATPases

It has been argued that all ATPases or even all the enzymes using ATP evolved from a common ancestral gene (Walker *et al.*, 1982; Pedersen and Carafoli, 1987). It was based on the presence of the sequence GXXXXGKT in polypeptides containing an ATP-binding site. The addition of several more

sequences to the list has diminished the conserved sequence to GXXXXG, and the sequence homology was replaced by a structural equation that may not be related to the evolution of the various polypeptides involved in ATP-binding (Taylor and Green, 1989). On the other hand, there are families of enzymes that maintain high homology among their members and appear closely related to other families. The V-ATPase family and the F-ATPase family clearly show such a relation. Although both of these enzymes function in proton pumping and protonmotive force-driven ATP formation and they show structural similarities, they clearly can be distinguished as two separate families. The ATP-synthase of archeabacteria is much closer to the vascuolar H^+-ATPases of the eukaryotic cells than to the ATP-synthase of eubacteria, chloroplasts, and mirochondria, and therefore the enzyme from archaebacteria was classified as V-ATPase (Nelson, 1988; Denda *et al.*, 1988a, b; Nelson and Taiz, 1989; Nelson and Nelson, 1989). Recent sequence analysis of genes encoding subunits of V-ATPases and comparisons with the wealth of sequence information available for F-ATPases generated surprising new information pertaining to both the evolution of these two families of proton pumps as well as the origin of eukaryotic cells.

The most important information came from the sequences of subunits *A* and *B* of the archaebacteria *Sulfolobus acidocaldarius* (Denda *et al.*, 1988a, b). Not only did Denda *et al.*, show that this ATP-synthase is a V-ATPase, but they also gave important clues for the evolution of proton pumps. Like the other V-ATPase, the largest subunit *A* (denoted as "α" in the original paper) is homologous to the β subunits of F-ATPases, and the smaller subunit *B* (denoted as "β") is more homologous to the α subunit of F-ATPases. The universal sequence for ATP-binding site of GXXGXGKT that is present in the β subunits of F-ATPases was conserved in all of the *A* subunits of V-ATPases including that of the archaebacterial enzyme (Zimniak *et al.*, 1988; Bowman *et al.*, 1988a; Denda *et al.*, 1988a). Analysis of the homologies among these subunits clearly showed that the β subunits of F-ATPases and the *A* subunits of V-ATPases evolved from a common ancestral gene.

Alignment of sequences of the *B* subunits of V-ATPases with those of subunits of F-ATPases gave a less clear distinction concerning the relation with the α or β subunits, but may shed light on the early evolution of the two families of proton pumps. Since the first sequencing of the operon encoding the F-ATPase in *E. coli*, it became apparent that certain stretches of amino acids within the α and β subunits are homologous (Walker *et al.*, 1982, 1984; Futai and Kanazawa, 1983). It was suggested that the α and β subunits of F-ATPase evolved by a gene duplication of a common ancestral gene. Similarly, alignment of the amino acid sequences of the *A* and *B* subunits of V-ATPase from *Neurospora crassa* and *Sulfolobus acidocaldarius* showed

Fig. 3. Alignment of stretches of amino acids with the highest homology among the *B* subunits of V-ATPases from *Saccharomyces cerevisiae* and *Sulfolobus acidocaldarius* and the α and β subunits of F-ATPase from *E. coli*. Published sequences are aligned using a DNASTAR program. The identical amino acids are indicated by asterisks. The positions of the first and the last amino acid of each stretch are indicated.

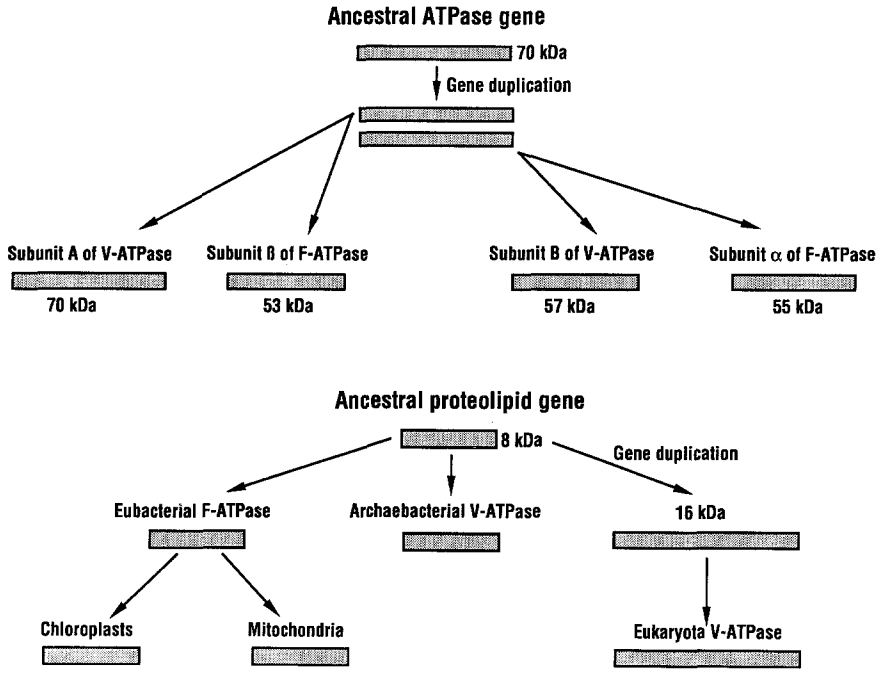

Fig. 4. Schematic proposal for the evolution of the primordial catalytic and membrane sectors of H^+-ATPases.

stretches of homology between the two subunits (Bowman *et al.*, 1988b; Denda *et al.*, 1988b). Figure 3 depicts the most homologous parts of the *B* subunits of V-ATPases from yeast and *Sulfolobus*, in relation to highly homologous stretches of amino acids of the α and β subunits of *E. coli* F-ATPase (Nelson and Nelson, 1989). The later sequences align best to the same position of the amino acid sequences of the *B* subunits of the V-ATPase. Even the overall percentage of identity between the α or β subunits of F-ATPase and the *B* subunit of V-ATPase is similar (Manolson *et al.*, 1988; Nelson *et al.*, 1989). Taking these observations together, it is likely that all four subunits, α and β of F-ATPases and *A* and *B* of V-ATPases, evolved from a common ancestral gene. The current gene that maintains the closest relation to the ancestral gene is the *B* subunit of the present V-ATPases (Bowman *et al.*, 1988b; Nelson and Nelson, 1989). Figure 4 (top) shows a schematic proposal for the evolution of the two large subunits of F and V-ATPases. It is suggested that the ancestral H^+-ATPase gene of about 70 kDa contained the ATP-binding site of GXXGXGKT that is currently present in the *A* subunits of V-ATPases and β subunits of F-ATPases. However, it was more homologous to the current *B* subunit of V-ATPase

than all the other subunits of the present proton pumps. The primordial enzyme was composed of a hexamer of the ancestral gene product, and a membrane sector for proton conduction. The ancestral gene underwent duplication, and the two genes evolved separately (Fig. 4, top). Three copies of each gene product comprise the catalytic sector of the enzyme. While one of the duplicates evolved into the *A* subunit of V-ATPase and the β subunit of F-ATPase, the second evolved into the *B* subunit of V-ATPase and the α subunit of F-ATPase. Subunit *A* of V-ATPase evolved through multiple changes of amino acids through the entire polypeptide, but no drastic change in its size took place. The β subunit evolved by deletion of over 100 amino acids from the middle of the parental gene and major changes in the other residues of the polypeptide. Subunit *B* evolved through small changes that included delection of stretches of amino acids and modification of the ATP-binding site. Similar change in the ATP-binding site took place in the evolution of the α subunit, with much more drastic changes in the amino acid residues. Comparisons of conserved regions between the various subunits suggests the identity of changes which occurred.

Subunit *E* is the only other subunit of the catalytic sector that was sequenced (Hirsch *et al.*, 1988). No sequence homology could be detected with either of the subunits γ, δ, or ε of F-ATPases. This may suggest that subunits δ, ε, *D*, and *E* were added later to the proton pumps in order to improve their function as ATP-synthases or in controlled acidification of organelles in eukaryotic cells.

The bulk of the membrane sector of the V-ATPase is composed of six copies of the proteolipid (Arai *et al.*, 1988). The genes encoding the proteolipids of the enzymes from chromaffin granules and yeast vacuoles were cloned and sequenced (Mandel *et al.*, 1988; Nelson and Nelson, 1989). The sequence information revealed that the proteolipids of V-ATPases from eukaryotic cells evolved by gene duplication of an ancestral gene. Figure 4 (bottom) depicts a schematic proposal for the evolution of proteolipids (DCCD-binding proteins) of proton pumps. It was proposed that the ancestral proteolipid gene was of the short version of about 8 kDa, currently present in F-ATPases (Hoppe and Sebald, 1984; Schneider and Altendorf, 1987). A proteolipid of this size is present not only in F-ATPases but in all the enzymes which function in ATP-synthesis, including the V-ATPase of archaebacteria (Denda *et al.*, 1989). On the other hand, all of the V-ATPases of eukaryotes have a molecular mass of about 16 kDa (Sutton and Apps, 1981; Manolson *et al.*, 1985; Bowman and Bowman, 1986; Randall and Sze, 1986; Arai *et al.*, 1987a; Sun *et al.*, 1987). It was proposed that the gene duplication that generated the larger version of the proteolipid was a major step in rendering the ancestral ATP-synthase into a controllable proton pump that can no longer function in ATP synthesis (Mandel *et al.*, 1988; Nelson and Nelson, 1989).

The available sequence information makes possible a preliminary reconstruction of the progenote's membrane H^+-ATPase and points out some of the event that led to the structure and properties of the current proton pumps (Maloney and Wilson, 1985; Harold, 1986). As early as we can identify, these enzymes were composed of a peripherical catalytic sector and a hydrophobic sector which function in transporting protons across the membrane. The catalytic sector was probably composed of six copies of an ancestral gene that later underwent gene duplication and each one of the new gene products was then present at three copies per enzyme. The primordial membrane sector was probably composed of six copies of proteolipid (8 kDa) to form a symmetric configuration with the hexameric catalytic sector. The next step was the addition of a single copy of an equivalent of the present γ subunit to the catalytic sector concomitantly with the addition of an equivalent of the *a* subunit to the membrane sector. This step preserves the symmetry of the enzyme. The available sequence data, especially the lack of information concerning the *C* subunits of V-ATPases, render it impossible to suggest whether the latter step took place prior to or after the segregation of F- and V-ATPases. The available sequence information does suggest that the progenitor of the catalytic sector of both families of H^+-ATPases was closely related to the current V-ATPases (Nelson and Nelson, 1989).

The evolution of the archaebacterial ATP-synthase involved small changes in comparison with the prognote's enzyme. Subunits *A* and *B* evolved together with their corresponding subunits of V-ATPases form eukaryotes. Subunit *C* was identified in the enzyme from *Sulfolobus* but its relation to the *C* subunit of the eukaryotic V-ATPase awaits sequencing of this subunit from the various sources. The proteolipid of archaebacteria is of the short version and its sequence should reveal some hints on the nature of the related subunit of the progenote.

The evolution of the progenote's ATP-synthase into the V-ATPase of eukaryotic cells involved small changes in the subunits' composition and amino acid sequences. The gene duplication of the proteolipid and the formation of a larger polypeptide may have been the most significant event in the conversion of the enzyme from an ATP-synthase into an enzyme that functions solely as a proton pump. Several additional changes took place in order to introduce the fine control of limited acidification of the various organelles (Moriyama and Nelson, 1988b). As for the archaebacteria, the sequence information of subunits *C* and *D* may reveal the origin of these polypeptides. The accessory polypeptides Ac115 and Ac39 are late additions of the vacuolar enzymes of eukaryotes.

The evolution of F-ATPases involved a chaoetic event that took place very early, and it was probably one of the most important events that shaped up the eubacteria. The main chaotic event was the deletion of a long stretch

of amino acids in the middle of the progenote's ATPase gene to create the β subunit of the newly developed F-ATPase. Concomitantly, the α subunit emerged up from the second duplicate of the primordial gene (see Fig. 4). These events released the new genes from their tight conservation and allowed more versatility found in the current F-ATPases in comparison with the present V-ATPases. Some of the genes encoding F-ATPase subunits in the organelles of the primitive eukaryotes were transferred to the nucleus. Several polypeptides were added to the complex, especially to that of the mitochondrial F-ATPase.

The antiquity of H^+-ATPase makes them attractive for evolutionary studies; their complexity seasons it, and their vital role in nature raises the desire to know them.

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