Biochimica et Biophysica Acta, 1100 (1992) 109–124

BBABIO 43605

Review

Evolution of organellar proton-ATPases

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(Received 19 November 1991)

Key words: Evolution; ATPase, H^+-; Vacuolar system; Chloroplast; Mitochondrion

Contents

Abstract

Proton ATPases function in biological energy conversion in every known living cell. Their ubiquity and antiquity make them a prime source for evolutionary studies. There are two related families of H^+-ATPases; while the family of F-ATPases function in eubacteria chloroplasts and mitochondria, the family of V-ATPases are present in archaebacteria and the vacuolar system of eukaryotic cells. Sequence analysis of several subunits of V- and F-ATPases revealed several of the important steps in their evolution. Moreover, these studies shed light on the evolution of the various organelles of eukaryotes and suggested some events in the evolution of the three kingdoms of eubacteria, archaebacteria and eukaryotes.

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I. Introduction

Eukaryotic cells contain a wide variety of organelles that function in many vital processes. All of these organelles maintain an interior composition different from that of the cytoplasm, and consequently they require a constant supply of energy to maintain their structure and concentration gradients of solutes across their membranes. Chloroplasts and mitochondria provide their own energy by the processes of photosynthesis and respiration. They also provide the bulk of the

energy requirements of the cell including the organelles of the vacuolar system. Proton-ATPases play a major role in both processes of ATP production and its utilization by the vacuolar system for driving secondary processes. There is a clear distinction between the semiautonomous organelles which contain their own unique DNA and RNA molecules; and the rest of the organelles that are connected to the vacuolar system which contain no unique DNA. F-ATPases are exclusively present in chloroplasts and mitochondria because they require organellar gene products for their functional assembly. In contrast, V-ATPases are composed only of nuclear gene products and are present in organelles of the vacuolar system and in the plasma membrane. V-ATPases are related in their structure and mechanism of action to F-ATPases, but they have sufficient unique properties to warrant placing them into separate families [1–3]. The most fundamental difference between these two families of proton pumps is a consequence of their function and origin. While the main function of F-ATPases is to synthesize ATP at the expense of protonmotive force (pmf) generated by electron transport chains, the main function of V-ATPases in eukaryotic cells is to generate pmf at the expense of ATP and to cause limited acidification of the internal space of several organelles of the vacuolar system. The origin of F-ATPases is rooted in the eubacteria that evolved into chloroplasts and mitochondria [4,5]. The origin of V-ATPases is related to archaebacteria, and therefore the evolution of the two families of proton pump is highly relevant to their structure, function and mechanism of actions [3].

II. Structure of F- and V-ATPases

Most of the membrane proteins are engaged in vectorial processes and the organization of their subunits in relation to the membrane is highly relevant to their function [6–8]. All of the ion pumps are catalyzing vectorial transport and the proton pumps are prime examples for such enzymes. In enzymes that are involved in fundamental processes, both amino-acid sequences and structures are highly conserved through evolution. Even though the conservation of amino-acid sequences captured most of our attention, it is important to remember that nature conserves structures of proteins without an apparent conservation in their amino-acid sequences.

The structure of F-ATPases has been reviewed in several articles, books and reviews [7–20]. On the other hand, only recently was the structure of V-ATPases elucidated [1,2,21–36]. Both F- and V-ATPases are multisubunit protein complexes that are built of distinct catalytic and membrane sectors. However, the precise number of subunits in some of these protein complexes is in the eye of the beholder and may change according to the definition of the object. A protein complex can be defined as the minimal structure that can catalyze a well-defined biochemical reaction. Consequently, several gene products that may be necessary for the correct assembly of the enzyme are excluded from their structure, and only those subunits that are present at stoichiometric amounts in the purified complex are considered as integral parts of the enzyme. Thus, the product of gene I in the *E. coli*

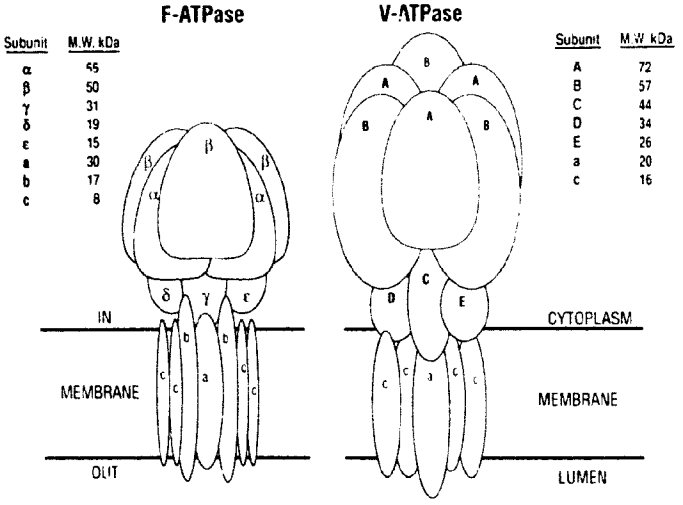

Fig. 1. Schematic depiction of the basic structure of F- and V-ATPases.

operon and the recently discovered assemblers of the yeast enzyme are not considered to be part of the respective F-ATPases [37]. Only the subunits of F-ATPase from *E. coli* and, to a lesser extent, those of chloroplasts were studied rigorously enough by resolution and reconstitution to show the necessity of each of the subunits for the activity of the enzyme as an ATP-dependent proton pump [38–48]. The illusion that molecular biology can replace biochemical studies for determining the integrity of suspected subunits as a part of protein complex, has been crushed on the rocks of the specific chaperons that are required for the assembly of F-ATPases [37]. Therefore, we were left with the old notion that resolution and reconstitution studies are necessary for determining the role of a polypeptide as an integral part of a certain protein complex [49]. Today, we know that F-ATPases from *E. coli* and chloroplasts contain 8 and 9 subunits, respectively, and we are not certain about the precise number of the mitochondrial F-ATPase subunits. However, the catalytic sector of all of these enzymes is composed of five subunits and only the membrane sector is still not entirely resolved.

The subunit structure of V-ATPases was studied in the last 5 years in several laboratories [21–36]. 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 [50–52]. Enzyme preparations from mammalian sources contained up to 13 different polypeptides [53]. Recent studies utilizing chaotropic agents and co'd inactivation in the presence of MgATP revealed that the catalytic sector of V-ATPases is composed of five different subunits that were denoted as subunits A to E in order of decreasing molecular mass from 70 to 26 kDa, respectively [1,21–23]. Fig. 1 depicts a schematic presentation of the basic subunit structure of F- and V-ATPases. The similarity between the basic structure of F- and V-ATPases is apparent, and functional homologs among the subunits were detected by nucleotide binding and chemical modification studies [51,52,54–58]. It was shown that A and β subunits contain the active sites of the respective enzymes and B and α subunits function in similar fashion [1–3]. The specific function of the other subunits of the catalytic sectors is not clear, and despite some very elegant and detailed studies of F-ATPases from *E. coli* and chloroplasts, only further studies may reveal their secrets [38–48].

The major subunit of the membrane sector is subunit c or 'proteolipid'. In both F- and V-ATPases it binds DCCD, and the binding prevents the ATP-dependent proton uptake activity of the enzymes [50–52,59–64]. The proteolipids from both families of proton pumps are very hydrophobic, soluble in chloroform/methanol solution, but those of V-ATPases are double the size (16 kDa) of those of F-ATPases. The membrane sector of the *E. coli* enzyme contains two additional subunits. Subunit a functions in proton conduction and subunit b functions in binding the catalytic sector and energy transduction between the two parts of the enzyme [63]. In chloroplasts subunits I to IV of CF_0 have comparable functions to subunits a to c of the *E. coli* enzyme [46–48]. So far there is no evidence of additional subunits in the membrane sector of V-ATPases. However, a very hydrophobic polypeptide of about 20 kDa was detected in a few preparations of V-ATPases [1,2,21–23,25]. It is tempting to speculate that this polypeptide may be related to subunit a of F-ATPases. Only cloning of the gene encoding this protein may show whether it is a subunit of the enzyme. Further information on the structure, function and evolution of H^+-ATPases came from recent cloning and sequencing of genes encoding various subunits of V-ATPases from different sources.

III. Molecular biology of F- and V-ATPases

The molecular biology of F-ATPases is only 10 years old, but it has yielded a large wealth of information. It started by the sequencing of the genes encoding the various subunits of the enzyme from *E. coli* [9,18,41,65–73]. These studies revealed that the genes encoding all the subunits of the enzyme are organized as a single operon in the bacterial genome [9,18,41]. The operon contains nine open reading frames, eight encoding the F-ATPase subunits and one (unc1) encoding a protein of unknown function. The order of the various genes in the operon is relevant to the structure and function of the enzyme. First comes *unc*I, encoding the protein that is not assembled into the enzymes, then there are the three genes encoding subunits a, c and b of the membrane sector followed by subunits δ, α, γ, β and ϵ of the catalytic sector. The sequence of the genes revealed that subunits α and β contain homologous sequences and they probably evolved from a common ancestral gene [18].

Sequence analysis and mutants generated by site-directed mutagenesis revealed a wealth of information on the function of individual subunits in the structure, assembly and catalytic activity of the enzyme from *E. coli* and the thermophilic bacterium PS3 [74–87]. The studies of enzymes from other sources is lagging behind. Most attention was devoted to the ATPase activity of the enzyme, but the proton translocation received its fair share [88–103]. I will mention only a few points of this extensive effort that are relevant for our discussion.

One of the most remarkable features of ATPases and other nucleotide binding proteins is the existence of a unique motif of amino-acid sequence called P-loop

or glycine reach sequence [18,104,105]. In *E. coli* F-ATPase β it consists of the sequence 138-PFAKG-GKVGLFGGAGVGKTV-157, and in subunit A of V-ATPase from yeast, PCVQGGTTCIPGAFGCGKT-V. Part of this sequence GXXXXGKT/S is common to all the nucleotide binding proteins and is currently used as a marker for such proteins. The three-dimensional structure of adenylate kinase and p21 ras protein have been solved and a common structure of the P-loop was revealed [106,107]. Since none of the three-dimensional structures of proton-ATPases was available, several groups used the kinase structure as a working hypothesis for the structure of P-loops in F- and V-ATPases [108–114]. However, a recent three-dimensional structure of F-ATPase from rat liver mitochondria somewhat reduced the value of this comparison [114a]. Site-specific mutagenesis in the β subunits of F-ATPase of the enzymes from *E. coli* and yeast revealed interesting features of the motif but did not resolve its function in the ATPase activity of the enzymes [115,116]. However, very important information on the flexibility of the P-loop structure was obtained. Surprisingly, most amino-acid replacements in the P-loop did not abolish the ATPase activity of the enzyme [76,78–85]. Swopping the glycine-rich portion of the p-loop between adenylate kinase and the β subunit resulted in an inactive enzyme. Remarkably, introducing the p21 ras sequence into the β subunit maintained about 50% of the original activity [114]. On the other hand, replacement of a few single amino acids in the corresponding region of the β subunit of yeast F-ATPase prevented the assembly of the enzyme [116]. However, replacement of Thr-197 to Ser-197 resulted in a 3-fold increase in the specific activity of the enzyme and altered sensitivity to oxyanions and NaN_3. It may be concluded that the structure of the P-loop is very important for the activity of ATPases and other nucleotide binding proteins, but the function of individual amino-acids may vary from one enzyme to another. The conserved sequence of GXXXXGKT/S in nucleotide binding proteins was taken as an indication that all of these polypeptides evolved from a common ancestor [117]. However, it is more likely that nucleotide-binding loops have evolved independently more than once [49,105].

The second amino-acid sequence that is important to note is the motif that binds phosphate in P-ATPases [115,118,119]. The aspartic residue in the sequence SVICSDKTG was shown to be phosphorylated as a reaction intermediate during catalytic turnover. The β subunits of F-ATPases contain an homologous sequence that in *E. coli* is 282-ITSTKTGSITS-292. In P-ATPases there is D instead of T-285 that binds the phosphate. In some of the A subunits of V-ATPases there is a glutamic acid residue in an analogous position [120,121]. In yeast aspartic acid is present at the same position [122]. One of the common properties of P-ATPases is their sensitivity to vanadate that is a consequence of forming phosphoenzyme-intermediate during catalysis. F-ATPases are not that sensitive to vanadate, and introducing aspartic acid in the corresponding position of the *E. coli* β subunit did not render the enzyme sensitive to vanadate, nor did it form a phosphoenzyme intermediate [81]. The polypeptide that incorporates phosphate during ATP hydrolysis in an enzyme from *Methanococcus voltae* is probably not a V-ATPase subunit [123]. This inconsistancy cannot rule out the possibility that P-, F- and V-ATPases share a common mechanism for coupling the ATPase and proton transport activities. Only discovering or engineering phosphoenzyme intermediate in F- or V-ATPase may support such an hypothesis.

The membrane sector of F- and V-ATPases functions in the proton transport activity of the enzyme. The mechanism of proton conduction is a total enigma, and despite numerous suggestions there is no agreement in any aspect of the process. Reconstitution studies with the enzyme from *E. coli* indicated that all three subunits of the membrane sector are required for proton translocation [63]. However, inspection of the sequences of these polypeptides suggested that only subunits a and c may be directly involved in the process [93–101]. Chemical modifications and studies with site-directed mutagenesis supported this notion [63]. Subunit c is a very hydrophobic protein that folds in the membrane like a hairpin with two membrane-traversing α-helices, and its polar loop extends from the cytoplasmic face of the membrane [124,125,126]. The reagent DCCD inhibits the ATP-dependent proton transport activity of all the F- and V-ATPases tested so far. It binds to a glutamic acid in the middle of the last trans-membrane helix of the various proteolipids [10,22,62,63,100]. In *E. coli* an aspartic acid residue is present at the same position. Extensive studies with site-directed mutagenesis were performed with the proteolipid (subunit c) of the *E. coli* F-ATPase. Most of the mutations were localized at or near aspartic acid 61, which is the DCCD-binding residue in this polypeptide [97,101,102,126–130]. Except for glutamic acid, all replacements of this residue rendered the enzyme inactive as a proton pump. Several DCCD-resistant mutants were localized in the vicinity of aspartate 61 mostly on the opposite transmembrane helix [99]. Remarkably, replacement of alanine 24 by aspartic acid suppressed the mutation of aspartate 61 to glycine that was inactive in proton conduction [131]. This interesting mutation shows that, even though a negative charge in the middle of the membrane is vital for proton pumping, its location can be changed between the two transmembrane helices of the proteolipid. The proteolipid of V-ATPases is much more conserved than that of the F-ATPases and conse-

quently more sensitive to changes in its amino acids [132]. It appears that here, too, a negative charge supplied by glutamic acid 137 is necessary for the function of the enzyme as a proton pump. Several changes in the vicinity of this residue in the proteolipid from yeast resulted in inactivation of the enzyme. Successful replacement of some amino acids by glycines suggested the possibility that this residue functions in proton conduction by attracting water molecules into the membrane [132]. No further studies by site-directed mutagenesis of V-ATPases have been reported so far.

The a subunit of F-ATPase from *E. coli* and several of its homologs from bacteria, chloroplasts and mitochondria contains charged and polar amino acids in the two transmembrane helices in its C-terminal end [10]. Changing arginine 210, glutamate 219 or histidine 245 caused inhibition, and several other mutations in this vicinity rendered the mutants oligomycin-resistant [93,96,133–136]. It was argued that the protons are conducted through the membrane via the charges of subunits c and a that are situated inside the membrane. So far there is no direct proof for such a mechanism, and the only system that comes close to providing evidence for such a mechanism is the proton conductance through a charge-relay in lac permease [137].

The extensive research of F-ATPases fails to reveal the mechanism of action of these proton pumps. There is a wealth of information on the mechanism of the ATPase activity of the enzyme [9,10,115], and some suggestions concerning the proton conduction across the membrane and several hypothetical approaches to explain the coupling between these two processes [138]. A picture is emerging indicating that the two processes may be coupled by a mechanochemical action in the enzyme. According to this mechanism the energy is conducted by conformational changes that take place along the enzyme, and by so doing communicate between the ATPase site and the proton conduction segment that are far apart. This coupling may require very flexible polypeptides such as the synthetic polypeptides that swell and contract by changes in their environment [139,140]. It is worth noting that glycine residues are playing a crucial role in providing the special properties to the synthetic polypeptides, and glycine residues are the most conserved abundant amino acids in F- and V-ATPases [132,141].

IV. Early events in the evolution of F- and V-ATPases

One of the dilemmas in discussing evolution is how far we can rely on amino-acid and nucleotide sequence homologies. If we insist on specific sequence identity such as 25%, we may discard valuable information. On the other hand, relying on identity among a few amino acids may lead to erroneous conclusions. It was argued

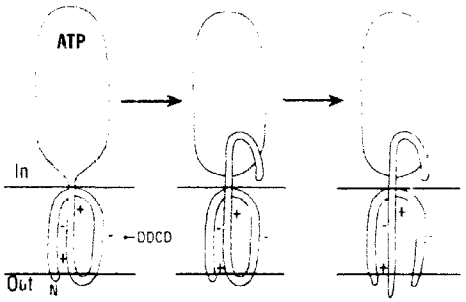

Fig. 2. Possible initial events in the evolution of H^+-ATPases that functioned in ATP synthesis.

that all of the proteins that bind ATP evolved from a common ancestral polypeptide [117]. This notion was challenged by others (see Ref. 105), and we consider this negative view more constructive for our discussion. For example, it will be difficult to prove whether P-ATPases evolved from a common ancestor with the F- and V-ATPases. On the other hand, recent sequence analysis of V- and F-ATPases revealed that they most probably evolved from a common ancestor [1,142]. All the enzymes of these two families contain distinct membrane and catalytic sectors with several subunits in each one of the sectors. It is assumed that today's protein complexes were evolved from much simpler structures and may be composed of a single gene product. If this is the case for V- and F-ATPases, it is quite likely that their primordial enzyme was composed of a polypeptide with a large globular portion with a small appendix of a very hydrophobic segment. Fig. 2 depicts such a polypeptide that represents both the membrane and the catalytic sectors. Since the hairpin loop of the current proteolipids is facing the catalytic sector [124], it is proposed that the primordial membrane segment contained three transmembrane helices, two of them having properties of today's proteolipid and the third containing other features of the current subunit a. Due to the conserved structure of three α and three β in F-ATPases, and three A and three B in V-ATPases, it is likely that the primordial enzyme had a hexameric shape. Very early in the evolution of the enzyme the membrane and catalytic sectors were separated into two different gene products and evolved separately ever since (see Fig. 2). Subsequently, subunits a and b were added to the complex to provide better binding and energy transfer between the two sectors of the enzyme. Imagination is the only support for such events and one may draw several alternatives for this scenario. The next steps in the evolution of F- and V-ATPases are witnessed in the sequences of the current enzymes.

Sequencing of the operon encoding F-ATPase in *E. coli* revealed that certain stretches of amino acids

within the α and β subunits are homologous [18]. It was suggested that the α and β subunits of F-ATPases evolved by gene duplication of a common ancestral gene. Subsequent sequencing of α and β subunits from a variety of sources supported this notion. Similarly, alignment of amino-acid sequences of the A and B subunits of V-ATPases from *Neurospora crassa* and *Sulfolobus acidocaldarius* showed stretches of homology between the two subunits [120,143–145]. All the A and B subunits of V-ATPases sequenced so far show the same property [121,122,146–150]. Inspection of the amino-acid sequences of the α and β subunits of F-ATPases with the A and B subunits of V-ATPases revealed that the four genes encoding these subunits evolved from a common ancestral gene [1,120–122,142–150]. Fig. 3 depicts a scenario of the early events in the evolution of the catalytic sectors of F- and V-ATPases. According to this proposal, the catalytic sector that was just separated from the membrane sector was compose of a hexamer of a single gene product. The ancestral gene underwent duplication, and the two genes evolved separately. Three copies of each gene product comprise the catalytic sector of the enzyme. While one of the duplicates evolved into the β subunit of F-ATPase and the A subunit of V-ATPase, the second evolved into the α subunit of F-ATPase and the B subunit of V-ATPase. That way, two classes of catalytic subunit were established, one involved more in catalysis and the other in regulating the activity of the enzyme. Up to this time there is no distinction between F- and V-ATPases. The separation into two families probably came with the introduction of new gene products into the enzyme. Sequencing of the gene encoding subunits C and E of various V-ATPases showed no homology with corresponding subunits of F-ATPases [151,152]. Even though the γ and ϵ subunits of F-ATPases evolved faster than the α and β subunits and consequently are less conserved, it is unlikely that the rate of evolution of the C and E subunits of V-ATPases was so fast as to eliminate residual sequence homology. We concluded that the γ and C subunits of the corresponding enzymes evolved from unrelated ancestral genes [152]. Subsequent addition of gene products further separated the two families of proton pumps and served specific functions in the different enzymes of various organisms.

The evolution of the membrane sector paralleled that of the catalytic sector. The genes encoding analogous proteins to the a and b subunits of F-ATPases were not found so far in V-ATPases. The only gene product that was positively identified in the membrane sector of all the V-ATPases is the proteolipid that binds DCCD and is soluble in chloroform/methanol solution [62,148,153]. In all the V-ATPases that were isolated from eukaryotic cells the size of this polypeptide is about 16 kDa, which is double the size of the F-ATPase proteolipids. Several genes encoding proteolipids of V-ATPases from various sources have been cloned and sequenced (Refs. 148, 153–160; Futai, M., personal communication). The sequence information revealed that the proteolipids of V-ATPases from eukaryotic cells evolved by gene duplication and fusion of an ancestral gene resembling the gene of F-ATPases [153]. Fig. 3 depicts the early events in the evolution of

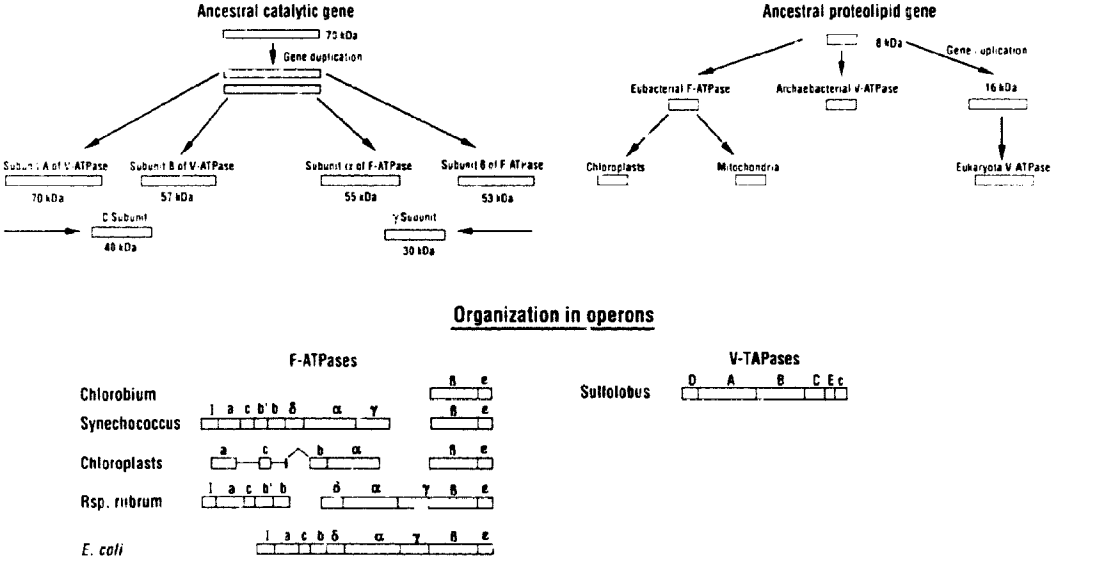

Fig. 3. Events in the evolution of the families of F- and V-ATPases and organization of genes encoding subunits of these enzymes in known operons.

the various proteolipids. One of the distinctions in the catalytic activity between F-ATPases and V-ATPases of eukaryotic cells is that the latter functions exclusively as ATP-dependent proton pumps and cannot form ATP at the expense of protonmotive force. On the other hand, V-ATPases of archaebacteria function in ATP synthesis. It was proposed that a proteolipid of the short version (about 8 kDa) is necessary for the activity of ATP formation [153]. Therefore, it was no surprise that the proteolipid of archaebacteria is of the short version [154–156]. It was proposed that one of the events that led to the evolution of V-ATPases of eukaryotes as exclusive proton pumps was the duplication of their gene encoding the proteolipid [1,148]. The structural evolution of proton pumps took part in generating effective machinery for energy coupling.

According to the scenario described above, the primitive proton pumps possessed radial symmetry just as primitive creatures. Advancement in evolution caused deviation from radial symmetry through bilateral symmetry into asymmetry. Molecular evidence to this notion is present in the atpI operon of cyanobacteria [161–164]. The membrane sector of F-ATPase from *E. coli* consists of 1 a, 2 b and 6–12 c subunits [165]. The presence of two b subunits in the membrane sector already imposes bilateral symmetry on the enzyme. In the atpI operon of cyanobacteria there are two genes encoding proteins that are homologous to the b subunit of *E. coli* (see Fig. 3). If indeed both of them are present in the F-ATPase of the cyanobacteria, the bilateral symmetry is violated. In chloroplasts of higher plants, both subunits I and II are homologous to the b subunit [166,167]. In this case it was shown that both subunits are present in the isolated F-ATPase from various sources [168]. The evolution of b subunit from bacteria to higher plants is written in the sequences of this subunit in different creatures. Fig. 3 depicts possible events in the evolution of the membrane sector of F-ATPases. Following the early events described in Fig. 2, the three fundamental subunits were evolved to form the membrane sector as present in the *E. coli* enzyme. Then the gene encoding subunit b underwent duplication into two separate genes that evolved independently as present in atpI operons of the current cyanobacteria. In higher plants one of this genes (encoding subunit II) was transferred to the nucleus, leaving the gene encoding subunit I in the chloroplasts. This evolutionary event rendered the chloroplast F-ATPase into an asymmetric enzyme. Recently, an astonishing experiment was reported where the gene encoding subunit I from chloroplasts successfully replaced the gene encoding subunit b in *E. coli* [169]. The two gene products contain very limited sequence homology. This experiment demonstrates that the structure of b subunits was strictly maintained while the amino-acid sequences underwent major changes. It also supports the notions that mechanochemical actions may be mediated by subunit b and for this action different properties than strict amino-acid sequences are more important for maintaining the activity of the enzyme.

Electron micrographs of isolated catalytic sectors also revealed asymmetry in its structure (170–172]. Fluorescent energy transfer studies among several binding sites on the chloroplasts enzyme demonstrated a deviation from symmetry in the structure of the catalytic sector [42,173–175]. Indeed, the presence of δ and ϵ subunits may be one of the reasons for the lack of ideal three-fold symmetry that is potentially present due to the organization of α and β subunits. The addition of other subunits to F-ATPases further shifted them out of symmetry, and the more advanced enzyme such as those of mammalian mitochondria must be assembled asymmetrically.

V. Operons of F- and V-ATPases

All of the genes sequenced so far encoding F- and V-ATPase subunits in bacteria are organized in operons. The most evolved operon is present in *E. coli*, in which all of the genes encoding the enzyme subunits are organized in a single operon [9,10,18]. There is a conserved order of genes in the operons of eubacteria and chloroplasts (see Fig. 3), and in all of the bacteria and chloroplast operons the β and ϵ genes are transcribed together. It is interesting to note that in all bacteria and chloroplasts that contain Photosystem-I-like reaction centers, the β and ϵ subunits are encoded in a separate operon [176]. In purple bacteria such as *Rhodospirilum rubrum*, the split is between the b and δ subunits and the genes encoding subunits of the membrane and the catalytic sectors are present in separate operons [177,178]. The mitochondrial and nuclear genes encoding F-ATPase subunits in eukaryotic cells are not arranged in operons. It is apparent that the genes in the archaebacterial V-ATPase operon are arranged in different order [160], and most probably evolved independently from the eubacterial ones.

One of the questions concerning the evolution of operons is what came first. Was the total operon such as that of *E. coli* formed first and only later was it broken into two operons and genes encoding single subunits? Or did the operon formation come late and evolve into various forms in different bacteria, and the total operon present in *E. coli* is a result of advancement in evolution? The first case is more convenient for discussing operons, but it seems as if the latter makes better sense. Recent sequencing of the operon encoding the β and ϵ subunits of F-ATPase in the green bacterium chlorobium revealed the presence of a separate operon encoding these subunits [176]. In light of the antiquity of these bacteria, it may be argued that

a split operon was present before the total operon. Even in chloroplasts the genes encoding some of the F-ATPase subunits maintained parts of the operon arrangement as present in *E. coli* [166,168]. The evolution of operons must have come after the presence of A, B and α, β subunits in the respective V- and F-ATPases was established. There are insufficient data to suggest the pathway of operon formation in archaebacteria. The operon encoding the β and ϵ subunits is present in all eubacteria and chloroplasts sequenced so far. This operon may have been the most primitive operon and for unknown reason it is present as a separate transcriptional unit in all the photosynthetic cells containing Photosystem-I-like reaction centers [176]. The advantage of the different operon structures is not clear and there is no evidence that the transcription of operons is better controlled than monocistronic genes. However, the existence of operons may have played an important role in the lateral transfer of genes among unrelated organisms. While evolution of proliferated families of genes may have benefited from exons flanked by large introns, the transformation of large structures from one organism to the other is easier when the genes are organizing in operons. Lateral transfer of large DNA fragments containing several genes encoding subunits of a protein complex is more likely than transferring the genes separately. This may have been the primary reason for the conservation of operons in bacteria and chloroplasts.

VI. Emergence and evolution of F-ATPase in chloroplasts and mitochondria

The endosymbiont hypothesis for the evolution of chloroplasts and mitochondria will never be proved beyond a shadow of a doubt. The lack of a finite answer is part of the weakness and beauty of evolutionary studies. This hypothesis had great influence on biological thinking, and therefore it is not relevant to our discussion if this hypothesis had been proven [5,179]. There is a wealth of evidence supporting the assumption that mitochondria and chloroplasts evolved by symbiosis between protoeukaryote and a primitive form of eubacteria. The structure, function, molecular biology and biogenesis of organellar F-ATPases presents the best evidence for that event. The structure of these enzymes is very similar regardless of their source [1,9,10]. Their function and mechanism of action are close enough to allow the replacement of subunits among enzymes of various sources [45,180–183]. All the F-ATPases in eukaryotic cells require organellar gene products for their functional assembly into ATP-dependent proton pumps. This phenomenon supports the hypothesis of the endosymbiontic origin of mitochondria and chloroplasts. Moreover, the transcription and translation of organellar genes resemble these reactions in eubacteria. The most likely time for establishing the endosymbiosis was when the oxygen amounts in the atmosphere rapidly increased due to the proliferation of organisms capable of light-dependent water oxidation. The protoeukaryotes that were evolved in a reducing atmosphere could not survive the oxygen tension without establishing symbiosis with oxygenic bacteria. It is assumed that the chloroplasts were evolved from bacteria that were similar to the current cyanobacteria or prochlorone [5,184–188]. The genome of these bacteria contains two operons encoding F-ATPase subunits. The operon atp1 includes the genes encoding the protein I and the subunits a, c, b′, b, δ, α and γ, respectively, and atp2 subunits β and ϵ. During the evolution of the chloroplasts' genome, the genes encoding subunits b′, δ, and γ were transferred to the nucleus. The presence of intermediate evolutionary steps in today's organisms make the evolution of the chloroplast genomes written in their DNA. For example, recently it was demonstrated that the gene encoding the δ subunit is present in the chloroplast genome of the diatom *Odontella sinensis* [189]. The more we look into chloroplast and mitochondrial genomes the more we are likely to find existing intermediate steps of their evolution.

Evolutionary dating of ribosomal RNA indicated that mitochondria may have evolved from photosynthetic purple bacteria such as *Rhodospirilum rubrum* [190]. As shown in Fig. 3 the two atp operons of *R. rubrum* are divided such that atp1 encodes subunits of the membrane sector and atp2 encodes only subunits of the catalytic sector. In mammalian and fungal F-ATPases the genes encoding subunits of the catalytic sector are present in the nucleus and only genes encoding some of the membrane sector are present in the mitochondrial genome [191–193]. This can be readily explained assuming the transport of atp2 operon of the purple bacteria into the nucleus as a unit. It is interesting to note that the gene encoding the α subunit is present in the plant mitochondrial genome [194]. This may represent a rudiment of ancient step in evolution but it is also conceivable that this gene was reintroduced to the plant mitochondria either from the nucleus or even from an unrelated organism. The latter proposal is consistent with the high rates of recombination in the plant mitochondrial genome [195]. The gene encoding the mitochondrial proteolipid is present in the nucleus of mammalian cells [196]. In fungi the gene is part of the yeast mitochondrial genome, but in *Neurospora crassa* it is transcribed from a nuclear gene [197]. It is interesting that the in mitochondrial genome of *N. crassa* there is a gene that has the potential for being a proteolipid gene, but it is not transcribed to the corresponding mRNA [198]. The dual location of genes encoding F-ATPase subunits in eukaryotic cells present a challenging coordination between the nuclear

and organellar genomes. Therefore, studies on the biogenesis of protein complexes including F-ATPases in chloroplasts and mitochondria captured the attention of several laboratories.

Assuming initial symbiosis between autonomous eubacteria and protoeukaryote, several major events should have happened during the evolution of organelles. The first step may have been the establishment of interdependency by eliminating metabolic processes that were duplicates in the two or three (algae) partners. This event can take shape by the inactivation of redundant genes. Next, appropriate transport systems should have introduced for metabolite transport among the compartments of the cell. The subsequent major step may have been the transfer of genes from the organelle into the nuclear genome. Concomitantly a protein-transport system should have been developed for import of nuclear gene products into the organelles. There are several indications that indeed these events took place during the evolution of mitochondria and chloroplasts. Recently utilizing the technique of DNA-coated tungsten particle bombardment for transformation of yeast mitochondria, it was demonstrated that DNA escapes from mitochondria and appears in the nucleus at a frequency at least 100 000-times higher than that of the DNA journey in the opposite direction [199]. Following the escape from the organelle, the DNA is readily taken up from the cytoplasm into the nucleus [200–202]. The mechanism of DNA escape from organelles is not known; it may take place by specific mechanism for DNA export, or more likely during terminal degradation of organelles by the vacuolar system of the eukaryotic cell. Thus, the feasibility of gene transfer from organelles into the nucleus was clearly demonstrated.

The evolution of the protein transport system is not very clear. A mechanism explaining the direction of the protein transport, which is opposite to the protein export by eubacteria, presents an intellectual challenge. Several aspects relevant to the evolution of the protein import into organelles were elucidated in the last few years. It was shown that the import of proteins into organelles requires energy in the form of electric potential and/or ATP [192,193,203]. It also requires a recognition system that includes receptors on the organelle's surface and signal sequences in the imported proteins to be recognized by the receptors. In most cases the signal sequences for the transport into organelles are N-terminal extensions with rather redundant features. The signal sequences usually contain amphiphilic helices, a common structure in proteins [204–206]. It was demonstrated that up to 5% of random DNA fragments that cloned in frame with DNA encoding cytoplasmic protein were competent as signal sequences for import into yeast mitochondria [207]. This seemingly sloppy design of signal sequences made it very easy for the newly transferred genes in the nucleus, to fuse with DNA fragments that are placed down stream of eukaryotic promoters and can be transcribed and translated into competent signal sequences. The new nuclear gene thus can produce a protein that is competent for functioning inside the organelle. Following transport into the organelle, most of the signal sequences are removed by proteolytic cleavage [191–193]. The proteinases that remove the signal sequences are very specific and they would not cleave any mature protein [208,209]. Therefore, the border between the signal sequence and the mature protein must have undergone considerable changes to make them competent for cleavage by the organellar proteinases. Conversely, evolutionary pressure should have been applied on the part of the genes encoding the mature protein to prevent them from having cleavable amino-acid sequences [210]. The latter may be a key element in understanding the evolution of signal sequence processing.

In an elegant set of protein uptake experiments with isolated yeast mitochondria it was shown that globular protein cannot be transported into the mitochondria [211–213]. It was demonstrated that the precursor protein should be maintained in its unfolded conformation before its uptake into the organelle takes place. This effect is obtained by molecular chaperones that are present in the cytoplasm of every eukaryotic cell [213–216]. Moreover, molecular chaperons inside the organelles play a major role in the import and assembly of the precursors into functional proteins [213–216]. Today's scenario for the mechanism of protein transport into the organelles is as follows: A precursor protein is usually synthesized by free polysomes and exposes its signal sequence into the cytoplasm. A chaperon binds the nascent chain preventing the protein from folding. ATP in the cytoplasm is required for the proper activity of chaperon. The complex of chaperon-precursor is recognized by the organellar receptor and a process of import is started. In some cases electric potential negative inside is required for the initial steps of the import process. When the precursor crosses the membrane, another molecular chaperon is required for keeping the protein in a proper conformation for its assembly. ATP inside the organelle is required for its activity, and it is likely that part of the energy provided by ATP is used for transporting the precursor into the organelle [203,217,218]. Next, the cleaved mature protein is assembled into its functional conformation as a monomer or part of a large protein complex. This simple description of protein import into organelles did not take into account the different compartments within the organelles.

Mitochondrial proteins have to be assembled in four different locations: the outer and inner membranes, the membrane interspace and the matrix [192,215].

Chloroplasts contain three different membranes and three different spaces to which protein should be imported and assembled into functional complexes [219–221]. It was demonstrated that proteins destined to the space between the inner and outer mitochondrial membranes contain an extended N-terminal signal sequence that is gradually cleaved by two different proteolytic enzymes. The first part of the signal sequence is cleaved by a soluble proteinase in the matrix and the second part is cleaved by an inner membrane-bound proteinase facing the interspace. There are two possible mechanisms for the import of such a protein. First, the precursor is initially transported into the matrix utilizing the first part of the signal sequence and then is exported into the interspace between the inner and outer membranes by a mechanism resembling the bacterial protein export system [222–224]. The second possibility is that the precursor is not transported into the matrix and only the first part of the signal sequence is present in the matrix and is cleaved by the matrix proteinase. After this cleavage, the precursor that is hooked on the external face of the inner membrane by the second part of the signal sequence is laterally moved to its position in the interspace [191]. The membrane-bound protein is then cleaved to give the mature protein, which can be assembled into its functional form in the interspace. Recently, the gene encoding the membrane-bound proteinase was cloned and sequenced [209]. The amino-acid sequence of this proteinase was found to be homologous to the leader peptidase of *E. coli* [222–224]. This finding indicates that parts of the protein secretion system of eubacteria are still present in mitochondria functioning in some aspects of the protein import system.

How has the polarity of protein transport been reversed in the organelles in relation to its direction in eubacteria? There is no clear answer to this question and it is quite possible that the import system had evolved by a mosaic assembly of parts from nonrelated systems. The second possibility is that it was acquired as a unit from another organism that established symbiosis with the protoeukaryote but lost all of its genes to the nucleus. It was proposed that peroxisomes evolved that way [225,226]. They contain a unique protein transport in which proteins are imported into the organelle without cleavable signal sequences [227,228]. The other candidate is an invisible archaebacteria that established endosymbiosis long before the current eukaryotes took shape, and left their mark not by a distinct DNA but instead by distinct systems such as the V-ATPases [3,229]. Assuming the system of protein import into organelles evolved from this ancient symbiosis the polarity of the organellar protein import is not a problem any more. The rudimental membrane systems evolved from this symbiosis are structured in eukaryotes to face the cytoplasm in the same configuration as they were in the archaebacteria. Therefore, the protein export of that archaebacteria could have been evolved into the organellar protein import system without changing its polarity. Evidence

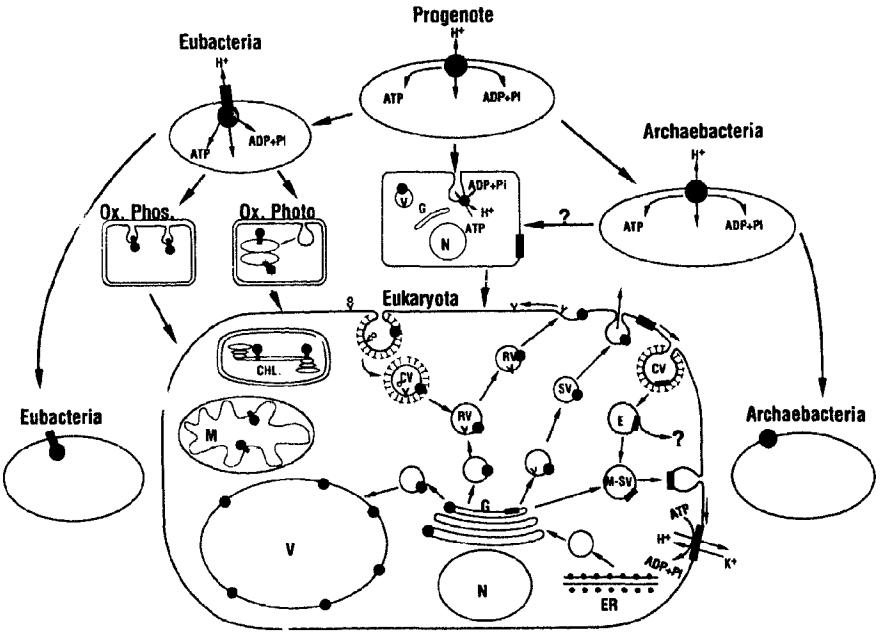

Fig. 4. Steps in the evolution of eubacteria, archaebacteria and eukaryota reflected by the evolution of F- and V-ATPases. (●), V-ATPase; (▮), F-ATPase; (▮), P-ATPase.

for such an evolutionary connection should be looked for in the protein export machinery of the current archaebacteria

VII. Evolution of V-ATPases in organelles of eukaryotes

It is assumed that the early atmosphere was reducing in nature and only the evolution of oxygenic photosynthesis drastically changed it into an oxidizing environment [230–232]. The amino-acid composition of V-ATPases suggests higher sensitivity to oxygen than their relatives F-ATPases. This observation prompts us to suggest that the catalytic sector of V-ATPase preceded that of the F-ATPase and the latter evolved from the former [1,148,233]. According to this hypothesis depicted in Fig. 4, the catalytic sector was composed of a hexamer of an ancestral gene product of about 70 kDa that was closely related to the current A subunit of V-ATPases. The ancestral gene underwent duplication and the two genes evolved separately to give gene products related to the A and B subunits. The enzyme was vulnerable to oxygen by virtue of its cysteine residues in the ATP-binding site and numerous tryptophan residues all over the subunits. In this form the enzyme could not cope with the increased oxygen tension brought about by the photosynthetic oxygen evolution. The β subunit of F-ATPase evolved from the ancestral gene product by deletion of over 100 amino acids from the middle of the polypeptide and major changes in the amino-acid composition, most notably substitution of most of the tryptophan residues [1]. The converse opinion – that the original ATPase subunit was closer to the subunit of F-ATPase and subunit A of V-ATPase evolved by addition of more then 100 amino acids in the middle – is also valid, though not very likely [3,121,142,149]. The V-ATPase now diverged into two distinct niches, cytoplasm of eukaryotes that is less susceptible to oxygen, and in archaebacteria that failed to evolve an F-type ATPase. The latter became more sturdy by substituting the cysteine residue in the active site with an amino acid that is less susceptible to oxygen.

Why do archaebacteria maintain the V-ATPase as their only ATP-synthase? The extensive studies of *Sulfolobus acidocaldarius* may partially answer this question [144,145,154,157,230–233]. This thermophilic bacterium grows at pH 2 and therefore its ATPase has to pump protons against concentration gradients of over 4 pH units. There is no report on F-ATPase capable of pumping protons against such a gradient. This is due to the stoichiometry of protons to ATP, that is equal to 3 in F-ATPases, and consequently at thermodynamic equilibrium a maximal ΔpH of less then 3 can be formed [234]. On the other hand, if V-ATPases operate with a proton to ATP stoichiometry of 2, ΔpH of over 4 can be established. Therefore, the V-ATPase operating in *Sulfolobus* is capable of maintaining ΔpH of over 4 units and the bacteria can grow at pH 2 [230–233]. The same property may be beneficial for growth at other extreme conditions such as high salinity. Several of the current archaebacteria inhabit niches with high environmental stress in which eubacteria and eukaryota are at a disadvantage.

The events that led the primordial V-ATPase into the vacuolar system of eukaryotic cells are not apparent. One of the most fundamental features of the enzyme in this system is that it operates below thermodynamic equilibrium [1,235]. It was proposed that a slippage of protons prevent it from reaching thermodynamic equilibrium and this slip is part of a mechanism involved in preventing the vacuolar system from undergoing overacidification. Therefore, it seems that nature utilized the less efficient enzyme (V-ATPase) by making it even less efficient for gaining better control over the extent of protonmotive force generated by the enzyme. The evolution of the V-ATPase may then have started by a symbiosis with a primordial archaebacteria or more likely by transformation and integration into the nucleus of a DNA fragment containing the genes encoding the ancestral enzyme. One of the first significant events in the evolution of the enzyme in the vacuolar system was probably the gene duplication and fusion of the proteolipid (DCCD-binding protein) [1,153]. This event was suggested to take place concomitantly with the development of the proton slip, and the transformation of the enzyme into an exclusive ATP-dependent proton pump that can no longer form ATP at the expense of protonmotive force. From this point, the enzyme should evolve to serve the ever growing number of specialized organelles in the vacuolar system. Like several other fundamental enzymes, the amino-acid sequences of the principal subunits in the catalytic and membrane sectors are very conserved. The proteolipids of V-ATPases contain two transmembrane helices that are nearly identical in yeast, *Drosophila*, plants, fish and mammals [148,155,156,159]. This is the most conserved hydrophobic protein present in the gene bank to date. The amino-acid sequence of subunit B is about 70% identical in the fungal, plant and mammalian enzymes and the same goes for subunit A [1–3]. Therefore, the most likely subunits that may play a role in diversification of the enzyme are subunits C, D and E of the catalytic sector and unknown proteins in the membrane. Accessory proteins may also play major role in this function [1]. In plants and mammals, isogenes encoding subunits A and B were reported (Ref. 149; Taiz et al., unpublished data). These isogene products in conjunction with isogenes encoding the less conserved subunits may provide the necessary diversification of the enzyme for serving specific functions in the various organelles.

The V-ATPase usually functions in internal organelles such as lysosomes and synaptic vesicles. However, in a few cases the enzyme functions in the plasma membrane of the cell secreting protons to its exterior [236]. It will be interesting to discover if specific gene products are taking part in the structure or assembly of these enzymes. The function of V-ATPases in a variety of organelles is a fine example of utilizing a conserved enzyme in numerous specialized organelles.

VIII. The evolutionary mosaic of organisms or the permissiveness of life

Sequence analysis of 16S rRNA from various sources suggested that a 'progenote' common ancestor diverged into three equidistant lines of descent: eukaryotes, eubacteria and archaebacteria [237]. An alternative evolutionary tree was recently proposed, in which the thermoacidophilic archaebacteria such as *Sulfolobus* are grouped with the 'eocytes', the line which gave rise to the eukaryotes [238]. At the same time an analysis of metazoan 18S rRNA sequences suggested a polyphyletic tree comprised of two groups that have originated from two separate ancestors [239,240]. This was inconsistent with 5S rRNA sequence data supporting a monophyletic tree with a common ancestor [241]. Can all of these suggestions be right and wrong at the same time? The answer may be yes, depending on the source of the sequences that one analyzes, and the method by which they were aligned [242]. Zillig et al. [229] analyzed several archaebacterial sequences and showed that, depending on the source of the sequence, different evolutionary relations can be observed. The presence of a number of 'eukaryotic' features in the archaebacteria has led to the proposal that the eukaryotes are more closely related to the archaebacteria than they are to the eubacteria [243–245]. However, the translation signals including ribosome-binding sites of the archaebacterial genome are similar to those of eubacteria. Moreover, archaebacterial genes can be organized in operons resembling those of eubacteria [229]. In contrast, the consensus sequence of archaebacterial promoters as well as the sequence of their DNA-dependent RNA polymerases closely resemble that of the eukaryotic RNA polymerase II. The simplest explanation for these observations is the assumption that during early evolution there was an extensive lateral movements of genes and large DNA fragments in organisms not necessarily related by family connection [246]. This early promiscuity helped to shape life and ever since has provided its essence. Close examination of amino-acid sequences of various proteins indicate that such a lateral gene transfer took place in a wide variety of organisms. In *Rhodobacter capsulatus* and *R. sphaeroides* most of the genes needed to provide a respiratory membrane with photosynthetic capability are clustered in giant operons [247]. It is quite likely that they were gained by lateral transfer from other organism. The classification of H^+-ATPases into F- and V-type where the F-ATPase is exclusively present in eubacteria, mitochondria and chloroplasts and the V-ATPase present only in archaebacteria and eukaryotes have recently gained its exception. Yoshida and his colleagues [248] have reported the isolation of an ATPase operon related to V-ATPases in the eubacterium *Thermus thermophilus*. This finding does not diminish the value of using H^+-ATPases for evolutionary dating [3,249]. It merely stresses the value of studying the evolution of various systems in different organisms for correct assessment of the origin of the specific organism in question.

The future of using organellar H^+-ATPases for evolutionary considerations is very bright, and those who ignore it are likely to pay the price with erroneous conclusions.

Acknowledgement

I wish to thank Dr. Michael J. Newman for critical reading of the manuscript.

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