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Subunit Composition, Structure, and Distribution of Bacterial V-Type ATPases

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The overall structure of V-ATPase complexes resembles that of F-type ATPases, but the stalk region is different and more complex. Database searches followed by sequence analysis of the five water-soluble stalk region subunits C–G revealed that (i) to date V-ATPases are found in 16 bacterial species, (ii) bacterial V-ATPases are closer to archaeal A-ATPases than to eukaryotic V-ATPases, and (iii) different groups of bacterial V-ATPases exist. Inconsistencies in the nomenclature of types and subunits are addressed. Attempts to assign subunit positions in V-ATPases based on biochemical experiments, chemical cross-linking, and electron microscopy are discussed. A structural model for prokaryotic and eukaryotic V-ATPases is proposed. The prokaryotic V-ATPase is considered to have a central stalk between headpiece and membrane flanked by two peripheral stalks. The eukaryotic V-ATPases have one additional peripheral stalk.

KEY WORDS: V-type ATPase; A-type ATPase; rotary catalysis; stalk subunits; stator structure; peripheral stalks.

INTRODUCTION

V-ATPases are membrane-bound rotary motor proteins. They function as proton- or sodium ion pumps to build up ion gradients at the expense of ATP and are widely distributed in different types of eukaryotic cells and some bacteria. V-ATPase consists of an extramembranous catalytic domain, called the headpiece V_1 that is linked by means of a stalk region to a membrane-bound ion-translocating domain called V_0. The mechanism of energy coupling between ATP hydrolysis in V_1 and ion translocation in V_0 involves the physical rotation of the central stalk in the complex. The prokaryotic V-ATPases are the more simple ones and consist of nine different subunits. The two large subunits A and B of the headpiece V_1 are each present in three copies in an alternating arrangement. Two other subunits, I and K, make V_0. They are known as "subunit a" and "subunit c" in eukaryotes, respectively. The remaining five subunits (C, D, E, F, G; in eukaryotes, "C" is termed "d") are considered to be part of the stalk region, together with the hydrophilic domain of subunit I (Nishi and Forgac, 2002). Eukaryotic V-ATPases have two additional subunits (H and a different subunit C), which are not present in the bacterial complexes. In addition, they contain two extra subunits, c′ and c" which are homologues of V_0 subunit c. In plants one other hydrophobic subunit, tentatively named VHA-e, is considered to be part of the V-ATPase (Sze *et al.*, 2002) and in some mammalian tissues, the enzyme contains an additional subunit termed Ac45 (Supek *et al.*, 1994).

No high-resolution structure of the complete V-ATPase or a subcomplex is available and neither do we know the positions of many of the nine different subunits that are common to the eukaryotic and bacterial complexes. In spite of the overall structural similarity of V-ATPases and F-ATPases, significant differences are observed especially in the stalk region. Early electron microscopy studies showed that the central stalk of V-ATPases was about 2 nm longer than in F-ATPases

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(Dschida and Bowman, 1992) and later studies indicated that the stalk region was more complex (Boekema *et al*., 1997). Electron microscopy (EM) images of V-ATPases revealed the presence of two or three peripheral stalks besides the central stalk (Boekema *et al*., 1999; Domgall *et al*., 2002), while F-ATPases are believed to contain a single peripheral stalk. The peripheral stalks are an essential part of the stator structure that prevents idle rotation of F_1 relative to F_0.

Scope of This Review

This paper attempts to summarize evolutionary, biochemical, and structural information of the bacterial V-ATPases in relation to their eukaryotic counterparts with the emphasis on the stalk region. For a better understanding of the differences between the complexes from different biological origins, it would already be useful to have a consistent low-resolution structural model. In particular the smaller subunits proposed to form the connection between the A_3B_3 headpiece and the membrane-bound c subunit ring structure seem to be the most relevant ones in this respect. The evolutionary relation between the five water-soluble stalk region subunits C, D, E, F, and G from bacteria, archaea, and the lower eukaryotes will be analyzed. Available structural data of V-ATPases mostly consists of two main bodies of information: (1) low-resolution studies by transmission electron microscopy and (2) biochemical data from cross-link studies. A substantial body of information about subunit positions has been obtained from the latter studies, but mainly on eukaryotic complexes. The most relevant data will be discussed here, together with some general features deduced from the amino acid sequences, as a starting point for composing a low-resolution model for the prokaryotic and eukaryotic V-ATPase complexes.

EVOLUTIONARY RELATIONSHIPS OF MICROBIAL V-ATPase STALK SUBUNITS

Database Searches

The main subunits of V-type and A-type ATPases, i.e., the headpiece subunits A and B, and the proteolipid subunit K are homologous to the corresponding subunits of F-type ATPases, indicating a common evolutionary origin and suggesting a similar mechanism of energy coupling by the complexes. Structural studies indicate differences between V-type ATPases and F-type ATPases especially in the stalk region (see below). Therefore, subunits C, D, E, F, and G may be diagnostic for V-type (and A-type) ATPase complexes. The distribution of bacterial V-type ATPases and their relation to eukaryotic V-type ATPases, archaeal A-type ATPases, and F-type ATPase was determined by BLAST searches (Altschul *et al.*, 1997) of the NCBI protein database (http://www.ncbi. nlm.nih.gov/blast/). Subunits C, D, E, F, and G of the *Enterococcus hirae* complex (Kakinuma, 1998) were used as the initial queries and all hits up to a specific Expect value were evaluated. All true positives were submitted to the BLAST server and the procedure was repeated until no new subunits were found. The procedure results in a set of sequences that are linked by (local) sequence similarity either directly or indirectly (see Table I). In the case of the F subunit of *E. hirae* the procedure converged very rapidly and only a single relationship (with the *Enterococcus faecium* subunit) was observed. The procedure was restarted using the VMA10 sequence of *Saccharomyces cerevisiae* which resulted in many more links between sequences, also in the bacterial kingdom. The relation between this set and the F subunits of the two *Enterococcus* species is based on analysis of the structure of the operons in which the genes are organized in bacterial and archaeal organisms. Since the ATPase complex operon structure in the bacterial and archaeal organisms is often conserved (see below), corresponding genes may be assigned on the basis of their position in the operon. The latter allowed for the further identification of a number of subunits that were not picked up in the BLAST searches (the sequences shown in Table I). This is usually only possible for organisms for which the complete genome sequence is available (http://www.ncbi. nlm.nih.gov/PMGifs/Genomes/micr.html) and it should be noted that the evolutionary relation is purely based on operon structure and not on amino acid sequence analysis.

Table I reveals the different nomenclature used for the locus coding for the ATPase complexes. The genes are termed *ntp*, *vat*, *atp*, and *vma*. The nomenclature is rather consistent for subunits C, D, and E, except for the eukaryotic fungi where they are numbered as *vma6*, *vma8*, and *vma4*, respectively. The C subunit of the fungi is termed subunit d and the D and E subunits of the crenarchaeota, γ and δ, respectively. The nomenclature for subunits F and G is more confusing as both identifiers are used for the two subunits. Subunit G of the bacterial *Enterococcus* species is subunit F in the archaeal A-type complexes and VMA7 or F in the eukaryotic V-type complexes. The *E. hirae* F subunit corresponds to the G, E, B, H, F, ε, or VMA10 in other organisms.

Distribution Over the Kingdoms of Life

An exhaustive search of the database revealed the presence of 43 different microbial V-type and A-type

Table I. Distribution of the C-F Subunits of Microbial V- and A-Type ATPases[a]

	Organism		C		D		E		F		G
A. *Bacteria*											
Firmicutes	*Enterococcus hirae*	NTPC	1171783	NTPD	1171784	NTPE	1171785	NTPF	1171786	NTPG	1171787
	Enterococcus faecium		22992681		22992677		22992682		22992685		22992680
	Streptococcus pyogenes	NTPC	15674361	NTPD	21909658	NTPE	21909653		21909650	NTPG	21909655
	Streptococcus pneumoniae	C	15901173	D	15901169	E	15901174		15901177	G	15901172
	Clostridium perfringens	NTPC	18310622	NTPD	18310618	NTPE	18310623		18310626	NTPF	18310621
	Clostridium thermocellum		23021509		23021513		23021508		23021505		23021510
	Clostridium tetani		28210703		28210707		28210702	G	28210699		28210704
			28211927		28211921		28211924				28203142
Fusobacteria	*Fusobacterium nucleatum*	C	19705059	D	9705054	E	19705060	G	19705063	G	19705058
Spirochaetes	*Treponema pallidum*		15639523	D	15639517	E	15639520		15639524	F	15639521
				D	15639419	E	15639415		15639416		
	Borrelia burgdorferi			D	15594438	E	15594442				
Chlamydiales	*Chlamydia trachomatis*			ATPD	15605027	E	15605031				
	Chlamydia pneumoniae			ATPD	15618014	E	15618010				
	Chlamydia muridarum				15835197	E	15835201				
Thermus/	*Thermus thermophilus*	VATC	12585450	VATD	12585444	VATE	12585449			VATF	12585451
Deinococcus	*Deinococcus radiodurans*		15805725		15805729		15805724		15805721		15805726
Thermotogae	*Thermotoga neapolitana*				24298777		24298774				24298773
B. *Archaea*											
Euryarchaeota	*Archaeoglobus fulgidus*	ATPC	11498764	ATPD	11498768	ATPE	11498763	E	11498758	ATPF	11498765
	Pyrococcus abyssi	ATPC	14521962	ATPD	14521958	ATPE	14521963		14521963	ATPF	14521961
	Pyrococcus horikoshii		14591714		14591709		14591715		14591718		14591713
	Pyrococcus furiosus	C	18976552	D	18976556	E	18976551	B	18976548	F	18976553
	Methanocaldococcus jannaschii		15668392		15668796		15668393		15668396		15668391
	Methanothermobacter thermautotrophicus		15678975		15678971		15678976		15678979		15678974
	Methanosarcina acetivorans	ATPC	20092949	ATPD	20092953	ATPE	20092948	H	20092945	ATPF	20092950
	Methanosarcina mazei	C	21226884	D	21226880	E	21226885	H	21226888	F	21226883
	Methanosarcina barkeri		23051712		23051708		23051713		23051716		23051711
	Methanopyrus kandleri	NTPC	20094451	NTPD	20095110	NTPE	20094450			NTPF	20094452
	Haloferax volcanii	VATC	2493105			VATE	2493110			VATF	2493113
	Halobacterium sp.	ATPC	15790975	ATPD	15790969	ATPE	15790976		15790979	ATPF	15790974
	Thermoplasma volcanium		13540881		13540885		14324262	H	3540886		13540882
	Thermoplasma acidophilum	C	16081188	D	16081192	E	6081187	NTPF	16082633	F	16081189
	Ferroplasma acidarmanus		22406533		22406529		22406534		22406528		22406532
Crenarchaeota	*Desulfurococcus sp.*	VATC	12585389	VATD	12585393	VATE	12585388			VATF	12585390
	Aeropyrum pernix			γ	14600683		14600686				14600687
	Sulfolobus solfataricus			ATPD	15897486	ATPE	15897483	ATPG	15897486	ATPF	15897482
	Sulfolobus tokodaii			γ	15921727	δ	15921724	ε	15921728		15921723
	Sulfolobus acidocaldarius			ATPG	152917	VATE	12585562				
	Pyrobaculum aerophilum		18312156		18312159						18312081
C. *Eukarya*											
Fungi	*Saccharomyces cerevisiae*	VMA6	6323480	VMA8	6320784	VMA4	173169	VMA10	6321829	VMA7	6321457
	Schizosaccharomyces pombe	d	19115149	D	19076013	E	19115844	G	19113621	F	19113468
	Neurospora crassa	VMA6	1718099	VMA8	2995849	VMA4	2493134	VMA10	1814380	VMA7	12585520
	Candida albicans			VMA8	2493133	VMA4	10720346				

[a] The sequences were submitted to the BLAST server at http://www.ncbi.nlm.nih.gov/blast/ and the hits in the list were analyzed for true positives up to Expect values of e-3. Blast searches of subunits C, D, and G were unfiltered, while the E and F subunits were filtered for low complexity regions and composition-based statistics. The subunits in the top line of the table follow the *E. hirae* nomenclature (Kakinuma, 1998; Takase *et al*., 1994). Sequences in red were added on the basis of the operon structure on the genome. The right part of each entry indicates the GI number in the NCBI protein database. The left part gives the gene name when not trivial, otherwise, the name of the subunit as indicated in the Definition line was given. Yellow shaded regions represent sequences that share >20% pairwise sequence identity with the *E. hirae* subunits.

ATPase complexes based on homology searching of subunits C, D, E, F, and G (Table I). The identification of one of the subunits was taken as evidence that a full complement of V-type ATPase subunits is coded on the genome of an organism even though not all the subunits were found in the search. It is considered unlikely that homologues of the subunits would function independently or in combination with other proteins in different biologically active molecules. The 43 complexes are distributed over 41 different organisms, 16 bacteria, 21 archaea, and 4 lower eukaryotes. Two bacteria, the pathogens *Treponema pallidum* and *Clostridium tetani*, contain two different V-type ATPases. The four fungi represent a much larger group of higher eukaryotes that contain closely related V-type ATPases. In the lower eukaryotes, the V-type ATPases reside in the vacuoles where they have specialized functions, while F-type ATPases in the mitochondria are responsible for ATP synthesis. In archaea, the A-type ATPases are common and the only type of this class of energy transducers found. Moreover, all archaea sequenced today seem to have this type of ATPase/synthase. In bacteria, V-type ATPases are quite rare and most bacteria use F-type ATPases for chemiosmotic energy transduction. The V-type ATPases are most redundant among the low GC Gram-positives (Firmicutes), but also found in the phyla Fusobacteria, Spirochaetes, Chlamydiales, Thermus/Deinococcus, and Thermotogae. The high observed abundance in the Firmicutes is likely to be biased by the higher number of genomes sequenced in this phylum. It should be noted that even though V-type ATPases are found in two *Streptococcus* species and three *Clostridium* species, the presence of the enzyme is not common among these genera. The genomes of *Streptococcus mutans, Streptococcus agalactiae,* and *Clostridium acetobutylicum* do not harbor the coding genes. Since roughly 100 bacterial genomes have been sequenced to date including many from Proteobacteria, it may be concluded that V-type ATPases will not be found in the Gram-negatives of the phylum Proteobacteria.

Sequence Conservation

The level of sequence conservation differs among the different subunits of the V-ATPase complexes. Subunit D is the best conserved subunit followed by subunit G. Next, subunits C and E have about the same level of conservation, while F is clearly the least conserved among these subunits. Except for subunit F, the genes coding for the corresponding subunits of the complexes from the bacterial phylum Firmicutes form a closely related family. The ATPase complex of *Caloramator fervidus* (formerly *Clostridium fervidus*) that we have characterized biochemically and structurally (Boekema *et al.*, 1997; 1999; Chaban *et al.*, 2002; Höner zu Bentrup *et al.*, 1997; Ubbink-Kok *et al.*, 2000) but not genetically is likely to be in this cluster as well. The different levels of conservation of the subunits is evident from the average pairwise sequence identities in a multiple sequence alignment (ClustalX; Jeanmougin *et al.*, 1998) of the subunits in this cluster that were 28% (C), 56% (D), 22% (E), 20% (F), and 49% (G). Closest to the cluster of the Firmicutes is the complex of the oral bacterium *Fusobacterium nucleatum* from the phylum Fusobacteria. Bacteria from these two phyla share similarities in the main metabolic pathways (Kapatral *et al.*, 2002).

The D subunits from the Firmicutes have significant overall sequence identity with the D subunits of ATPase complexes from all three kingdoms of life (yellow, Table I) while the G subunits share high sequence identity with the corresponding subunits of many of the A-type ATPases. Moreover, subunits C and E from the Gram-positives revealed many hits in the BLAST searches with the archaeal proteins, but none with the eukaryotic V-type ATPases. It follows that the bacterial V-type ATPases are closer to the archaeal A-type ATPases than to the V-type complexes of the lower eukaryotic microbes, like *Saccharomyces cereviseae*. The link between the bacterial and eukaryotic V-type ATPase is made through the A-type ATPases which reveals some inconsistency in the nomenclature. This conclusion based upon the stalk subunits is in line with a phylogenetic analysis of the catalytic A subunit of V- and A-type ATPases published before (Hilario and Gogarten, 1998).

Remarkably, the Firmicutes subunits appear to be more close to many of the archaeal subunits than to the subunits of the complexes from the other bacterial phyla Spirochaetes, Chlamydiales, Thermus/Deinococcus, and Thermotogae, suggesting diversity among the bacterial V-ATPases. Again, with the limited set of data available at that time, the analysis of the catalytic A subunit already positioned the *E. hirae* and *T. thermophilus* on different branches of the tree (Hilario and Gogarten, 1998). The V-type ATPases of *E. hirae* and *C. fervidus* are known to operate in the ATP hydrolysis mode, like their vacuolar counterparts. Archaeal complexes may both function in the ATP hydrolysis and synthesis mode. Possibly, the evolutionary diversity in the bacterial V-type ATPase reflects these two modes of action. The reported ATP synthesis activity of the V-type ATPase of *Thermus thermophilus* in the phylum Thermus/Deinococcus would be consistent with such a scheme (Yokoyama *et al.*, 1998).

Subunit F of the bacterial complexes is only poorly conserved. No relation based on sequence could be

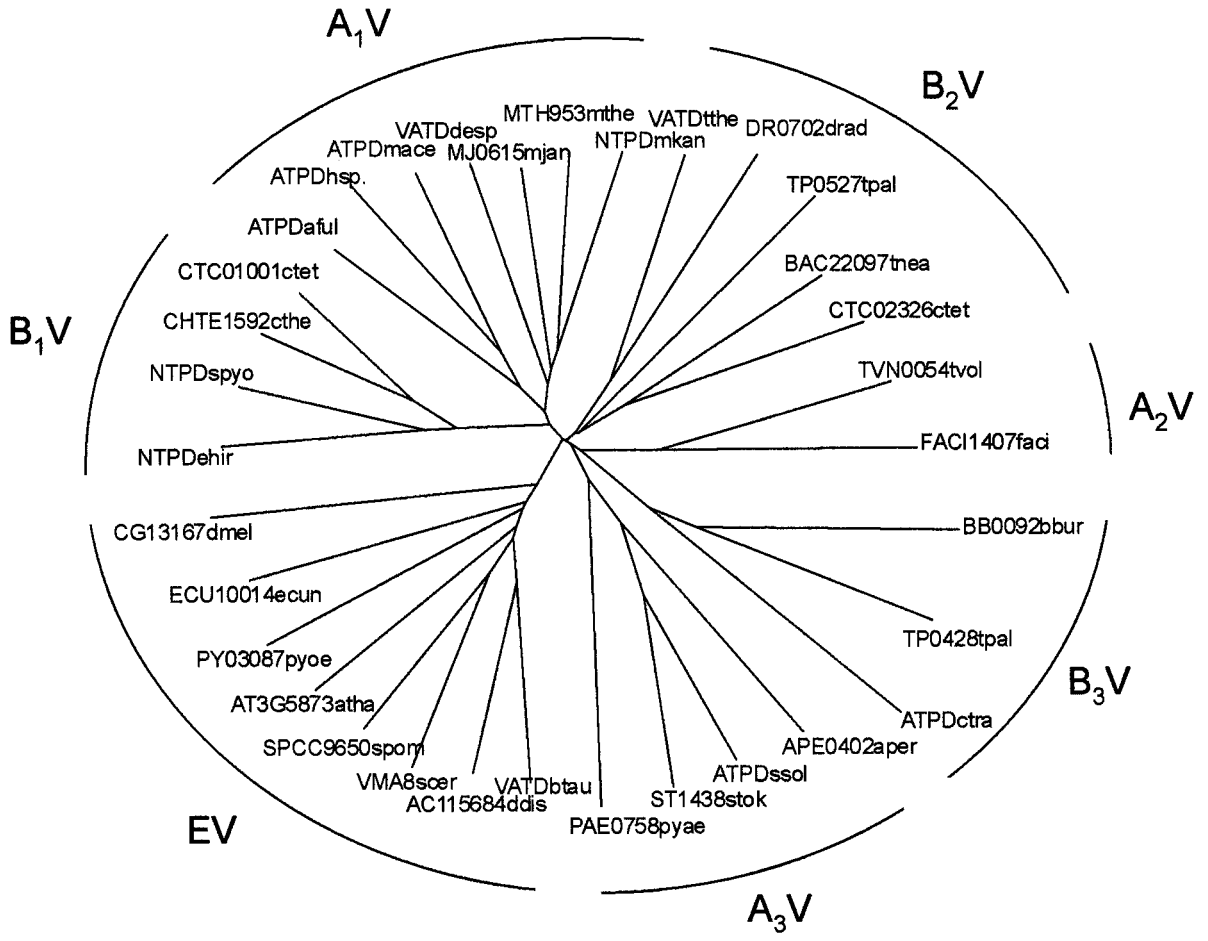

Fig. 1. Phylogenetic tree of the D subunits. The tree was constructed using the Drawtree program in the Phylip package (Felsenstein, 1993) after multiple sequence alignment using ClustalX (Jeanmougin *et al.*, 1998). Included in the alignment were the sequences listed in Table I, except for those that shared a sequence identity >60% with another sequence in the set. The gene names are followed by a shorthand for the organism that is composed of the first character of the genus followed by the first three characters of the species (for instance, ctet for *Clostridium tetani*). Genes TP0527 and TP0428 of *Treponema pallidum* correspond to GI numbers 15639517 and 15639419, respectively, and genes CTC01001 and CTC02326 of *C. tetani* to 28210707 and 28211921, respectively. In addition to the sequences from Table I, six sequences from higher eukaryotes were included to better position the eukaryotic subunits on the tree. These include subunits D of the V-ATPase complexes of *Bos Taurus* (btau), *Arabidopsis thaliana* (atha), *Drosophila melanogaster* (dmel), *Encephalitozoon cuniculi* (ecun), *Dictyostelium discoideum* (ddis), and *Plasmodium yoelii* (pyoe). The median of the pairwise sequence identity distribution after multiple sequence alignment was 26%.

established between the F subunits of *E. hirae* and *E. faecium* and the corresponding subunits from the other bacterial complexes. The low conservation is typical for the bacterial F subunits. For instance, the averaged pairwise sequence identity of all eukaryotic F subunits, including those of higher organisms, was 29%. Apparently, the structure and the interaction with the other subunits of the complex sets less high constraints on the amino acid sequence of the bacterial F subunit as compared to the eukaryotic F subunit. Eukaryotic V-ATPases are more complex structures involving subunits not present in the bacterial V-ATPases. Possibly, the F subunit is associated with these additional features of the eukaryotic enzymes and is rudimentary in the bacterial complexes.

A nice summary of the relation between the ATPase complexes from different sources is provided by a phylogenetic tree of the D subunits (Fig. 1). The highly conserved D subunits provide the most reliable multiple sequence alignment including proteins from all kingdoms of life. The eukaryotic microbial subunits cluster on a single branch of the tree that is well separated from the other branches (Fig. 1, EV). Subunits D of higher eukaryotes are on the same branch. The bacterial subunits are on three different branches. The Firmicutes and Fusobacteria are

on one branch (B_1V) as are the Thermus/Deinococcus, Thermotoga, and one of the *T. pallidum* subunits from the Spirochaetes (B_2V). The D subunit from the second complex of *T. pallidum* and the Spirochaet *B. burgdorferi* and the Chlamydiales are on the third branch (B_3V). Subunits D from the archaeal kingdom are also distributed over three branches. The Euryarchaeotal subunits reside on branch A_1V, except for the Thermoplasmas and *Ferroplasma acidarmanus* that are on branch A_2V. Finally, branch A_3V contains the Crenarchaeotal subunits. The bacterial branch B_1V and the archaeal branch A_1V are loosely associated. The subunits on bacterial branch B_3V are most distant in the whole set.

The BLAST searches using the C, D, E, and G subunits as queries revealed no hits with F-type ATPase subunits. In this respect these subunits are different from the A, B, and K subunits. The F subunit picks up the peripheral stalk subunit b of the F-type ATPases with high Expect values. Unfortunately, both sequences suffer from low complexity regions, which makes the biological significance of the observation questionable. Considering the size of the protein database and along with that the number of F-type ATPase sequences from different organisms in the database, it seems save to conclude that at least for the C, D, E, and G subunits of the V-type ATPases no evolutionary links to F-type subunits will be found. This indicates that either the corresponding subunits diverged below the level of detectable sequence similarity or the proteins evolved independently, i.e., are unrelated proteins.

Operon Structure of V- and A-Type ATPase Complexes

The genes coding for the subunits of the bacterial V-type and archaeal A-type ATPase complexes are organized in operon structures that are remarkably well conserved throughout the two kingdoms. The most frequently observed sequence of the genes is F-I-K-E-C-G-A-B-D (Table II). The conserved sequence allowed the tentative assignment of the poorly conserved F subunit to many hypothetical genes on the genome. They were assigned on the basis of their position in front of the gene coding for subunit I and the number of residues in the gene product. The above operon structure seems to be the rule in the phyla Firmicutes, Fusobacteria, Thermus/Deinococcus, and in the Euryarchaeota, except for the Thermoplasma species were the H (F) and I subunits have moved from the front to the end of the gene sequence. The conservation of the operon structure correlates with the close relation between the bacterial and archaeal D subunits in branches B_1V and A_1V (Fig. 1). Also in this respect, the Thermoplasma species seem to be the exception to the rule in the Euryarchaeota (branch A_2V), while the Thermus/Deinococcus species reside on branch B_2V. In the Euryarchaeota *Methanopyrus kandleri* and the *Halobacterium* species, the operon is split in two distant parts on the genome while the gene order is maintained. The genes coding for the B and D and for the D subunits, respectively, are located downstream of the other subunits with a considerable number of other genes in between the two parts.

The genomes of the Spirochaetes and Chlamydiales contain a different operon structure for the V-ATPase genes that is characterized by the sequence E-X-A-B-D-I-K-Y in which X and Y are putative genes that do not show homology to any protein in the database. In the Chlamydiales group, the X and Y proteins are homologous. No homologues of subunits C, F, and G in these bacteria were found. Possibly, X and Y have taken over the functions of two of these. On the genome of the three Chlamydia species, the operon is flanked on both sides by genes of known function suggesting that the V-type ATPase may consist of eight rather than nine subunits, missing the F subunit. This group of bacteria is found on branch B_3V in Fig. 1.

Among the Crenarchaeota the operon structure seems to be the least conserved. Moreover, all operons are split operons. The best conserved order seems to be I-F(G)-E-A-B-D. This order is also observed in the operon coding for the bacterial *T. pallidum* V-ATPase in branch B_2V.

SUBUNIT POSITIONS FROM BIOCHEMICAL STUDIES

In this part, interactions of subunits within the stalk region are summarized and discussed together with the most relevant biochemical data concerning their structural positions. The eukaryotic subunit nomenclature (C, D, E, F, G, H, a, c, d) was adopted in this part because the eukaryotic complexes contain the higher number of subunits. Eukaryotic subunits D, E, F, G, a, c, d correspond to *E. hirae* subunits D, E, G, F, I, K, and C, respectively, while eukaryotic subunits C and H do not have bacterial counterparts.

Subunit C

Subunit C is only present in eukaryotic V-ATPase complexes. Site-directed mutational studies on subunit C did not provide direct evidence for structural placement

Table II. operon Structures of Bacterial V-Type and Archaeal A-Type ATPases

	Organism	Gene order[a]	
A. *Bacteria*			
Firmicutes	*Enterococcus hirae*	F-	I-K-E-C-G-A-B-D
	Streptococcus pyogenes	SpyM3_0114[b]-	I-K-E-C-G-A-B-D
	Streptococcus pneumoniae	SP1323[b]-	I-K-E-C-G-A-B-D
	Clostridium perfringens	CPE1644[c]-	I-K-E-C-F-A-B-D
	Clostridium tetani	G-	I-K-E-C-G-A-B-D
			C-I-K-E-A-B-D
Fusobacteria	*Fusobacterium nucleatum*	G-	I-K-E-C-G-A-B-D
Spirochaetes	*Treponema pallidum*	TP0535[d]-TP0534[e]-	I-F-E-A-B-D
		E-TP0425[f]-	A-B-D-I-K
	Borrelia burgdorferi	BB0097[g]-E-BB0095[h]-	A-B-D-I-K-BB0089[i]
Chlamydiales	*Chlamydia trachomatis*	E-CT309[j]-	A-B-D-I-K-CT303[k]
	Chlamydia pneumoniae	E-CPn0087[j]-	A-B-D-I-K-CPn0093[k]
	Chlamydia muridarum	E-TC0583[j]-	A-B-D-I-K-TC0577[k]
Thermus/Deinococcus	*Thermus thermophilus*	G-	I-K-E-X-F-A-B-D
	Deinococcus radiodurans	DR0694[b]-	I-K-E-C-F-A-B-D
B. *Archaea*			
Euryarchaeota	*Archaeoglobus fulgidus*	AF1158[c]-	I-K-X-K'-E-C-F-A-B-D
	Pyrococcus abyssi	PAB1179[c]-	I-K-E-C-F-A-B-D
	Pyrococcus horikoshii	F-	I-K-E-C-F-A-B-D
	Pyrococcus furiosus	PF0176[c]-	I-K-E-C-F-A-B-D
	Methanocaldococcus jannaschii	MJ0223[c]-	I-K-E-C-F-A-B-||[l]-D
	Methanothermobacter thermautotrophicus	MTH961[c]-	I-K-E-C-F-A-B-D
	Methanosarcina acetivorans	H-	I-K-E-C-F-A-B-D
	Methanosarcina mazei	H-	I-K-E-C-F-A-B-D
	Methanopyrus kandleri		I-K-E-C-F-A-||-B-D
	Halobacterium sp.	H-	I-K-E-C-F-A-B-||-D
	Thermoplasma volcanium		K-E-C-F-A-B-D-H-I
	Thermoplasma acidophilum		K-E-C-F-A-B-D-H-I
Crenarchaeota	*Aeropyrum pernix*		G-E-A-B-D-||-K
	Sulfolobus solfataricus	I[m]-||-	F-E-A-B-D-G-K
	Sulfolobus tokodaii	I[m]-||-	G-E-A-B-D-STS172[n]-K
	Pyrobaculum aerophilum		F-E-A-||-C-||-D

[a] Subunits were indicated as annotated in the databases at http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html (see Table I for conversion). The operon structures of the *E. hirae* and *T. thermophilus* complexes were taken from Kakinuma (1998) and Yokoyama *et al.* (2000), respectively.
[b] Putative subunit F (*E. hirae* nomenclature) by position on genome.
[c] Putative subunit F (*E. hirae* nomenclature) by homology.
[d] Residues = 71.
[e] Residues = 342.
[f] Residues = 86.
[g] Residues = 129.
[h] Residues = 182.
[i] Residues = 325.
[j] Residues = 266-7, homologous protins.
[k] Residues = 173-3, homologous protins.
[l] || Indicates a discontinuous sequence.
[m] Subunit I located immediately in front of subunit F but on opposite strand.
[n] Homologous to subunit G of *S. solfataricus*.

of this subunit in the complex, but the phenotype of the mutants was consistent with the C subunit playing a role in the stator of the enzyme (Curtis *et al.*, 2002). The subunit does not tightly associate with the V_0 complex (Xu *et al.*, 1999).

Subunit D

Because subunit D is predicted to have a high α-helical content, it was suggested to be the subunit corresponding to the γ subunit of F-ATPase, which forms

the major part of the rotating central stalk (Xu *et al.*, 1999). But for the same and other reasons, subunit E could be the central stalk (Margolles-Clark *et al.*, 1999). Subunit D does not associate with a V_0 subcomplex. On the other hand, V_1 subunits failed to associate to the membrane in a yeast mutant lacking the D subunit (Graham *et al.*, 1995). Subunits D and E are at least part of the time close together, because they could be cross-linked by 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Xu *et al.*, 1999). Subunit D could also be cross-linked to subunit F by DSG (Xu *et al.*, 1999) to form a stable complex that was proposed to be able to associate with other V_1 subunits (Tomashek *et al.*, 1997). Subunits D and F and possible other stalk subunits seem to undergo conformational changes driven by redox modulation (Grüber *et al.*, 2000b).

Subunit E

Cysteine-directed cross-linking of subunit E to subunit B was possible, but only at sites on subunit B that are considered to be oriented to the outer surface of the complex (Arata *et al.*, 2002). This suggests that subunit E forms part of the peripheral stalk. Subunit E has also been reported to cross-link to C (Xu *et al.*, 1999), to F (Grüber *et al.*, 2000a), to G (Grüber *et al.*, 2000a; Xu *et al.*, 1999), and to the soluble domain of subunit a (Xu *et al.*, 1999). It can also interact with H (Lu *et al.*, 2002). Rather intact V_1 complexes can form in the absence of subunit E (Tomashek *et al.*, 1996). Subunit E appears to be more abundant than subunits C and D suggesting the presence of two copies per complex (Nelson and Harvey, 1999). In contrast to the work of the group of Forgac, Grüber *et al.* (2002) conclude that E is shielded in V_1 and rather a part of the central stalk. The fact that the E subunit can be labelled with a biotinyl-ATP compound could also point to a position of subunit E in the central cavity of the V_1 headpiece (Schäfer *et al.*, 2001). Electron microscopy studies on the *C. fervidus* complex also indicate that it forms the upper part of the central stalk (Chaban *et al.*, 2002).

Subunit F

Subunit F is a globular protein (Jones *et al.*, 2001). Although it does not show sequence similarity with the ε subunit of F-ATPase, both types of subunits have about the same pI and data suggest that they also share similarities in their folding (Jones *et al.*, 2001). In cells lacking subunit F, the V_1 subunits were unable to associate with V_0 (Graham *et al.*, 2000), suggesting that subunit F might be localized in the central stalk.

Subunit G

Supekova *et al.* (1996) and Hunt and Bowman (1997) first noticed that subunit G appears to be homologous to the hydrophilic part of the b subunit found in F-ATPases. But as discussed earlier, the similarity is not very strong. Subunit G likely cross-links with E (Xu *et al.*, 1999). The fact that it appears to be present in two copies per V-ATPase molecule (Xu *et al.*, 1999), like the b subunit in prokaryotic F-ATPase, could be an argument for a rather similar role in a peripheral, connecting stalk. This would also give an explanation for the observed low sequence homology (Table I).

Subunit H

Subunit H is only present in eukaryotic V-ATPases. It is different from subunit H in some archaeal complexes that corresponds to subunit F (see Table I). Subunit H is a rather large subunit with a mass of about 50 kDa. The structure of the H subunit from yeast has been solved by X-ray diffraction (Sagermann *et al.*, 2001). It has an elongated structure and is primarily α-helical. Subunit H has a direct interaction with subunit a (Landolt-Marticorena *et al.*, 2000) and could be cross-linked to subunits E and F (Xu *et al.*, 1999). It is likely that subunit H is located near the periphery of the complex because rather intact V-ATPase complexes could be isolated from a yeast mutant lacking the H gene product (see Xu *et al.*, 1999). In mammalian and yeast V-ATPases there are two isoforms of H, αSFD and βSFD, each present in one copy per complex as determined by quantitative amino acid analysis (Margolles-Clark *et al.*, 1999; Xu *et al.*, 1999; Zhou *et al.*, 1998).

Subunit a

The "a" subunit (or I in prokaryotes) of the V_0 domain has a bipartite structure containing a hydrophilic amino-terminal domain of up to 50 kDa and a hydrophobic carboxyl-terminal domain containing multiple putative transmembrane α-helices. Subunit a plays a crucial role in ion translocation (Kawano *et al.*, 2002) and the coupling between proton transport and ATP hydrolysis (Kawasaki-Nishi *et al.*, 2001). Thus, the V-ATPase a subunit corresponds to the a subunit of the F-ATPases, which functions as part of the stator that is held rigid relative to the $\alpha_3\beta_3$ F_1 hexamer (Xu *et al.*, 1999). There is evidence that the amino-terminal domain in yeast directly interacts with the H subunit (Landolt-Marticorena *et al.*, 2000).

Subunit c

Subunit c, also known as the proteolipid subunit, is a hydrophobic subunit with four membrane-spanning α-helices. It is assumed to be present in six copies in prokaryotes, which associate to form a ring structure that constitutes the rotor in the V_0 part. The rotor rotates in the plane of the membrane against the static a subunit. In eukaryotes, one or two copies are replaced by c' and c" subunits, which are homologous proteins with the same number of membrane-spanning α-helices (Nishi *et al.*, 2003). Models for the arrangement of the three subunits in the rotor structure have been proposed (Perzov *et al.*, 2001; Powell *et al.*, 2000). The actual arrangement, however, does not seem to be very crucial for the current assignment of the stalk subunits at low resolution.

Subunit d

Subunit d (named subunit C in all prokaryotes) is often ranked under the V_0 subunits, but it does not contain apparent transmembrane segments. Electron microscopy studies indicate that it forms a spherical density in the lower part of the central stalk, where it is connected to the center of V_0 (Chaban *et al.*, 2002). This position was already suggested from early cross-link studies (Adachi *et al.*, 1990).

STRUCTURAL DATA FROM ELECTRON MICROSCOPY AND IMPLICATIONS

On the basis of crystallographic analysis of images of two-dimensional (2D) crystals, it is possible to obtain medium-to-high resolution three-dimensional (3D) structures by transmission EM. A good example in the field of ATPases is the work of Stokes and colleagues on the Ca^{2+}-ATPase (Zhang *et al.*, 1998). Native 2D crystals of V-ATPase present in transporting epithelia have been imaged by EM (Brown *et al.*, 1987). Potentially, these could be suitable for image analysis, but such a crystallographic determination of the V-ATPase structure has not yet been carried out. All our knowledge about the overall structure of V-ATPase stems from single particle EM. Recently, four different groups have presented structural data on V-ATPases from different sources obtained by image processing. Two studies have presented 2D views of the complete molecule (Boekema *et al.*, 1999; Wilkens *et al.*, 1999), a third one also concerns a 3D reconstruction (Domgall *et al.*, 2002). One of the interesting features is the presence of a central stalk and two additional connections between V_1 and V_0 in prokaryotic V-ATPase (Boekema *et al.*, 1999). In the projection maps of V-ATPase from bovine brain clathrin-coated vesicles the number of stalks is not precisely clear, mainly because there is more mass present in the stalk region (Wilkens *et al.*, 1999). On the other hand, the 3D reconstruction of plant V-ATPase clearly shows three peripheral connections besides a central stalk (Domgall *et al.*, 2002). These data are not necessarily in conflict, because of differences in subunit composition and structure between the various V-ATPases studied. A comparison of the projection EM images from different V-ATPases is shown in Fig. 2.

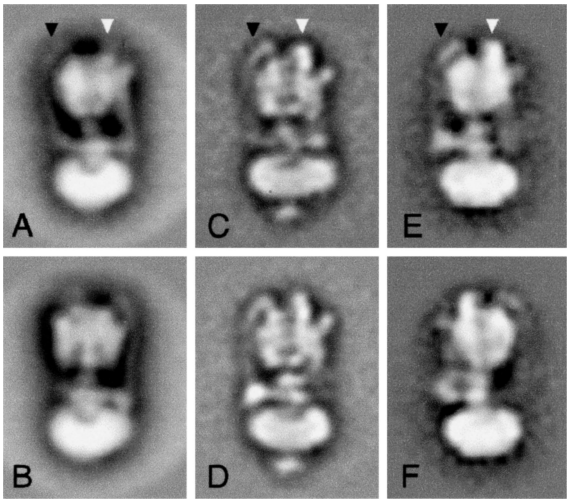

Fig. 2. Comparison of side-view projections from different V-ATPases. (A, B) Selected side views from *C. fervidus* (Ubbink-Kok *et al.*, 2000). (C, D) Views from bovine brain clathrin-coated vesicles (from Wilkens *et al.*, 1999, with permission). (E, F) Views from plant tonoplasts (from Domgall *et al.*, 2002, with permission). The frames A, C, and E show the headpieces in relatively similar positions, as can be seen from knob-like densities protruding from the A subunit on the right side of the headpiece. On the left side, similar-shaped connecting stalks are visible. Black arrowheads indicate the positions where these connecting stalks attach to the top of the headpiece. Masses protruding on the top right side of the headpiece (white arrowheads), are considered to be the end of a second connecting stalk. In the V-ATPases from bovine brain and plant tonoplasts this mass is much larger than observed in the *C. fervidus* complex. The V-ATPase from bovine brain shows the Ac45 subunit, as a protruding density at its base (images C, D).

In general, a single particle EM analysis often yields not only different average views from identical molecules in different positions, but also views of molecules that have lost one or more subunits. By automatic classification schemes, such fragmented molecules can be recognized and, subsequently averaged separately. The purified V-ATPase makes no exception to the rule that large multisubunit proteins may lose some of its subunits.

A comparison of the variations in averaged EM projections indicates a variable protein mass at the top of

the A_3B_3 headpiece moiety (Ubbink-Kok *et al*., 2000). It can be concluded that two different masses protrude from the top. This is clearly confirmed by the EM analysis and difference mapping of specific V_1 fragments (Chaban *et al*., 2002). It is likely that the two different masses at the top of the V-ATPase belong to two different subunits. This situation is somewhat similar to the F-ATPases. An analysis of about 17,000 side-view projections from freshly prepared chloroplast F-ATPase indicated the variable presence of one protein mass at the top of the F_1 headpiece (Van Roon *et al*., 2000). Antibody labelling showed this mass to be the δ subunit (Wilkens *et al*., 2000), which is present in just one copy. The fact that peripheral stalks bind to the top of V_1 in a way similar to the F-ATPases has been neglected in almost all V-ATPase models presented to date.

Top view projections of single V_1 headpieces from *C. fervidus* show the hexameric arrangement of the two times three copies of the A and B subunits. The A subunits appear larger than the B subunits (Boekema *et al*., 1998), because of a knob-like density, formed by a 10-kDa insertion at the N-terminal part of the subunit. This insertion is absent in the corresponding F-ATPase β-subunit. The knobs are especially prominent in top view images of slightly tilted headpieces and in one type of side view of the complex (Fig. 2; Chaban *et al*., 2002).

Depending on the subunit composition, an additional central mass may be present in V_1 headpieces corresponding to the central stalk (Chaban *et al*., 2002). Some V_1 projections of *C. fervidus* also show a peripheral mass at the interface of an A and B subunit (E. J. Boekema, unpublished data). Averaged top view projections of V_1 headpieces from the tobacco hornworm *Manduca sexta* showed the same features (Radermacher *et al*., 1999). A central density is within V_1, but also a peripheral density at the interface of an A and B subunit is visible. Basically four small subunits were present in the examined headpiece preparation (D, E, F, G). A recent model places them all in the central stalk (Rizzo *et al*., 2003), without giving a clue about the peripheral mass. We tend to interpret these molecules as having the central density formed by the E (and F?) subunits and the peripheral density by D (and G?).

TOWARDS A CONSISTENT MODEL FOR V-ATPase

The current biochemical and EM data do not allow for a consistent model in which all subunits can be positioned in an unequivocal way. Nevertheless we can present models for prokaryotic and eukaryotic V-ATPases that match the above-presented data as close as possible (Fig. 3). They show one central stalk, which is flanked by two peripheral stalks in prokaryotic V-ATPase and three in eukaryotic V-ATPase. We will discuss some further details and consequences here. EM data indicate that the central stalk is composed of mainly two separate protein densities. The upper one protrudes far into the V_1headpiece, and has a maximal length of about 85 Å (Chaban *et al*., 2002). The overall shape is similar to the γ subunit of the F-ATPases. It appears to be formed by the E subunit (Chaban *et al*., 2002, and E. J. Boekema, unpublished data). The assignment is at variance with recent cross-link studies (Arata *et al*., 2002), which point to subunit D at this position. It should be emphasized that EM determines directly the overall position of a subunit, in contrast to cross-linking, which determines just the position of one part of a protein. It is unlikely that both D and E are together in the central stalk, as proposed by Tomashek *et al.* (1996), given their size and the fact that subunit d is already unequivocally assigned to the central stalk. Moreover one other small subunit, likely F, is also considered to be located somewhere in the central stalk. The lower density of the central stalk is formed by the d subunit (C in prokaryotes) (Chaban *et al*., 2002). Since subunit d does not have a counterpart in F-ATPases, this explains why the central stalk of V-ATPase is substantially longer than the central stalk of F-ATPase.

Subunits D and G are the most likely candidates for one of the peripheral stalks. Possibly two copies of G would form most of the long rod-like connecting mass seen in EM images. The question then remains how this peripheral stalk is attached to the top of the headpiece. It could be in a way as it occurs in F-ATPases, as discussed earlier. Then, there should be a V-ATPase specific subunit equivalent to the F-ATPase δ subunit. Subunit D could be a candidate but the fact that it cross-links to several other small subunits makes this unlikely. Thus, probably, D is in the lower half of this peripheral stalk.

Subunit a (subunit I in prokaryotes) is a good candidate to be involved in a second peripheral stalk (Domgal *et al*., 2002). In their 3D model, the a subunit is proposed to fill one of the densities which extend from the membrane to the top of V_1, where it ends in a prominent protrusion (Fig. 2). The presence of one prominent protrusion (and also one with lower mass) is obvious from the 3D reconstruction, but also from projection maps of bovine clathrin-coated vesicle V-ATPase (Wilkens *et al*., 1999). However, the same prominent protrusion was not present in the side views of *C. fervidus* V-ATPase (Fig. 2; Ubbink-Kok *et al.*, 2000). This would be consistent with the substantially smaller mass of the bacterial subunit I vs. the eukaryotic counterpart subunit a. In eukaryotes and prokaryotes, its mass is 100 and 61 kDa, respectively.

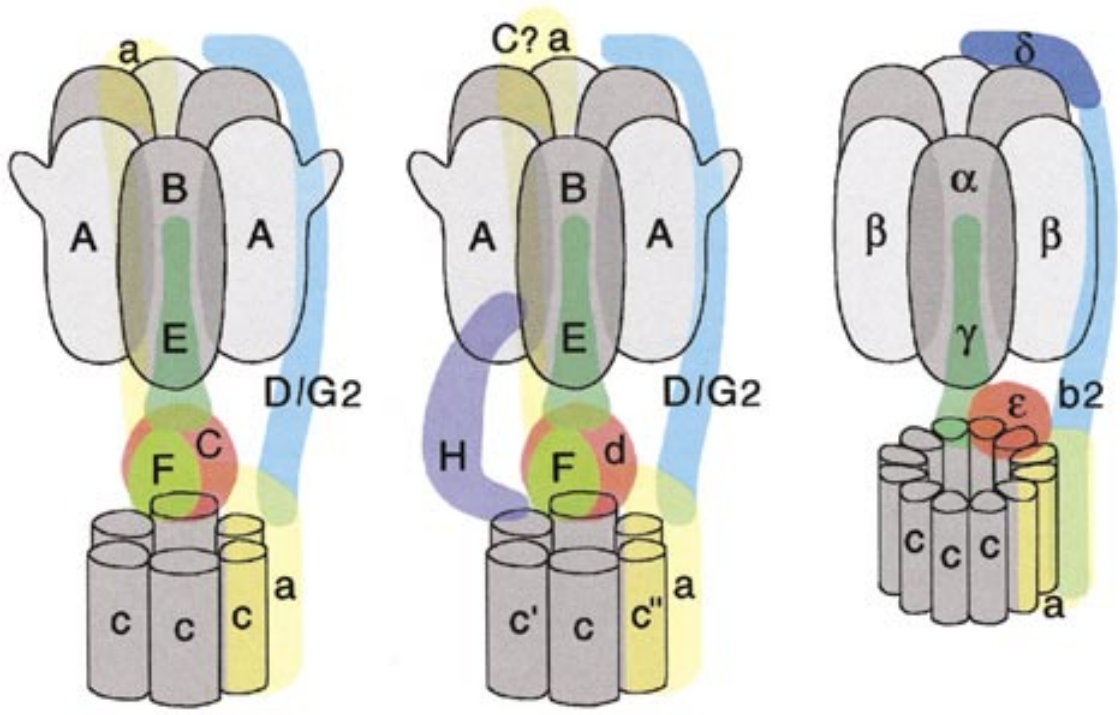

Fig. 3. Models for prokaryotic V-ATPase (left), eukaryotic V-ATPase (middle), and prokaryotic F-ATPase (right). The A_3B_3 headpiece and the subunit c multimer (all in grey) are connected by a central stalk, composed of subunits E and d/C and possibly F. Three peripheral stalks are built of subunit D plus G, subunit a (plus C?) and of subunit H. The latter connection is absent in prokaryotic V-ATPase. In the eukaryotic V_0, one or two copies of subunit c are replaced by the homologous subunits c′ and c″. *Note*: In the prokaryotic V-ATPase the lower central stalk subunit C is homologous to subunit d in eukaryotes and *not* related to the subunit C of eukaryotes. F and G used in this scheme correspond to the yeast genes VMA7 and VMA10, although in some prokaryotic systems the names have been exchanged.

Alternatively, the absence of the prominent protrusion in *C. fervidus* V-ATPase may be explained by the lack of another subunit at the top, possibly subunit C. However, there is no indication that this subunit is located on top of V_1. The interpretation that subunit a forms one of the peripheral stalk is not supported by a 3D reconstruction of isolated clathrin-coated vesicle V_0(Wilkens and Forgac, 2001). In this reconstruction subunit a does not extend far from V_0. It is considered to be responsible for two elongated densities of 7×3 nm and 6×3 nm, together with the d subunit (Wilkens and Forgac, 2001). However, it cannot be excluded that the conformation and/or position of subunit a may undergo drastic changes after removal of the V_1 headpiece.

Subunit H may function as the third peripheral stalk connecting V_1 and V_0 in eukaryotic V-ATPase. The evidence comes from the 3D reconstruction of plant V-ATPase. The high-resolution structure of subunit H fits well into one of the kinked connecting masses of the 3D reconstruction from electron microscopy (Domgall *et al.*, 2002). The fact that H is not present in prokaryotic V-ATPase would explain why the EM data from *C. fervidus* do not show three peripheral connections.

Finally, from the EM data, only some other specific features can be assigned. For instance, the protrusion at the base of V_0 of the V-ATPase from clathrin-coated vesicles must be the hydrophilic part of the Ac45 subunit, a 14th subunit present in some eukaryotic V-ATPases (Wilkens and Forgac, 2001). This density is absent in the EM data from plants and prokaryotes (Fig. 2).

CONCLUSIONS

The nomenclature of some of the subunits of the bacterial V-ATPase and archaeal A-ATPases is quite inconsistent and confusing. This is especially the case for the C, F, and G subunits. Since the eukaryotic complexes have the highest number of subunits and the bacterial and archaeal subunits appear to be a subset of these, it is recommended to adapt the eukaryotic naming of the subunits throughout the V-ATPases and A-ATPases. Evolutionary analysis

of the stalk subunits revealed that the V-type and A-type ATPases are the same type of ATPase. For historical reasons, we propose to name this type V-type ATPases and indicate the biological origin as EV for eukaryotic, AV for archaeal, and BV for bacterial V-ATPases (adopted in Fig. 1) following a similar scheme as is common for F-type ATPases (BF, CF, MF, . . .). Then, V-type ATPases would differ from F-type ATPases in the connection between the headpiece and the membrane embedded part. The difference is both in the architecture and the proteins that built the stalk region. Subunits d, D, E, and G would be diagnostic for V-ATPase; subunits b, γ, δ, and ε for F-type ATPases.

Phylogenetic analysis of the stalk subunits of the V-ATPase, together with the operon structure found in bacteria and archaea, suggests the following model for the distribution of V-ATPase. V-ATPases are rooted in the archaeal kingdom as it is coded on all sequenced archaeal genomes today. From there, the enzyme found its way into the eukaryotic world. In bacteria, F-type ATPases would be the standard. Bacterial V-ATPases were picked up by horizontal gene transfer from archaea, an event that may have happened multiple times during evolution (see also Hilario and Gogarten, 1998). As a result, V-ATPase stalk subunits in the phylum Firmicutes are closely related to the corresponding subunits in the euryarchaeota and they share the same operon organization. V-ATPases in other phyla appear to be more related to crenarchaeotic complexes. Some bacteria, *C. tetani* and *T. pallidum*, appear to have picked up two V-ATPase complexes from different sources.

The cross-linking data and other biochemical data concerning subunit interaction are in part contradicting. This indicates that a sound assignment of the stalk region subunits is not possible yet and that the models are still tentative. Further structural elucidation of the V-ATPase by electron microscopy is necessary. Comparisons of particles lacking specific subunits, as carried out by Chaban *et al*. (2002) could be extended. Another way to tackle the subunit topology would be to image particles with small metal (gold) clusters attached to specific subunit sites, as has been performed with other large membrane protein complexes. On the other hand, the ultimate goal would be to solve the full structure by X-ray diffraction. But it is not certain that V-ATPases will be stable enough to become crystallized with a complete set of peripheral stalk subunits. Probably a hybrid approach between X-ray and EM could be of interest as well. If EM could provide a 3D structure at medium-resolution ($\sim$8 Å), high-resolution components solved by X-ray diffraction could be fitted in. There are already a number of examples where this approach has been successful.

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