PHYSIOLOGICAL REVIEWS
Vol. 79, No. 2, April 1999
Printed in U.S.A.

Vacuolar and Plasma Membrane Proton-Adenosinetriphosphatases

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Nelson, Nathan, and William R. Harvey. Vacuolar and Plasma Membrane Proton-Adenosinetriphosphatases. *Physiol. Rev.* 79: 361–385, 1999.—The vacuolar H^+-ATPase (V-ATPase) is one of the most fundamental enzymes in nature. It functions in almost every eukaryotic cell and energizes a wide variety of organelles and membranes. V-ATPases have similar structure and mechanism of action with F-ATPase and several of their subunits evolved from common ancestors. In eukaryotic cells, F-ATPases are confined to the semi-autonomous organelles, chloroplasts, and mitochondria, which contain their own genes that encode some of the F-ATPase subunits. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form ATP at the expense of the proton-motive force (pmf), V-ATPases function exclusively as ATP-dependent proton pumps. The pmf generated by V-ATPases in organelles and membranes of eukaryotic cells is utilized as a driving force for numerous secondary transport processes. The mechanistic and structural relations between the two enzymes prompted us to suggest similar functional units in V-ATPase as was proposed to F-ATPase and to assign some of the V-ATPase subunit to one of four parts of a mechanochemical machine: a catalytic unit, a shaft, a hook, and a proton turbine. It was the yeast genetics that allowed the identification of special properties of individual subunits and the discovery of factors that are involved in the enzyme biogenesis and assembly. The V-ATPases play a major role as energizers of animal plasma membranes, especially apical plasma membranes of epithelial cells. This role was first recognized in plasma membranes of lepidopteran midgut and vertebrate kidney. The list of animals with plasma membranes that are energized by V-ATPases now includes members of most, if not all, animal phyla. This includes the classical Na^+ absorption by frog skin, male fertility through acidification of the sperm acrosome and the male reproductive tract, bone resorption by mammalian osteoclasts, and regulation of eye pressure. V-ATPase may function in Na^+ uptake by trout gills and energizes water secretion by contractile vacuoles in *Dictyostelium*. V-ATPase was first detected

in organelles connected with the vacuolar system. It is the main if not the only primary energy source for numerous transport systems in these organelles. The driving force for the accumulation of neurotransmitters into synaptic vesicles is pmf generated by V-ATPase. The acidification of lysosomes, which are required for the proper function of most of their enzymes, is provided by V-ATPase. The enzyme is also vital for the proper function of endosomes and the Golgi apparatus. In contrast to yeast vacuoles that maintain an internal pH of ~5.5, it is believed that the vacuoles of lemon fruit may have a pH as low as 2. Similarly, some brown and red alga maintain internal pH as low as 0.1 in their vacuoles. One of the outstanding questions in the field is how such a conserved enzyme as the V-ATPase can fulfill such diverse functions.

I. INTRODUCTION

Bioenergetics have been revitalized by new insights into the mechanism of energizing biomembranes. Structural information on electron transport complexes is becoming available, and the three-dimensional structure of the catalytic sector (F_1) of F-ATPase is being resolved (1). The large-scale DNA sequencing from various organisms revealed biological trends that previously were unknown to scientists (34, 57). The enormous knowledge available to the public through the Internet makes it possible to analyze complex systems with unprecedented sophistication. However, the fundamental mechanism of energy coupling remains largely obscure; its resolution may revolutionize our view of biological processes. Among the various advances in bioenergetics, two stories are particularly exciting: *1*) the emerging mechanochemical coupling between ATP hydrolysis and proton-motive force (pmf) and its utilization for driving numerous transport systems (1, 27, 99, 135), and *2*) vacuolar H^+-ATPase (V-ATPase) emerges as a pivotal player in the generation of pmf for this purpose. The story of V-ATPase started with studies on the energization of catecholamine uptake into chromaffin granules (105, 157). It was demonstrated that an ATPase energizes their membranes by an ATP-dependent proton uptake and that the proton gradient drives the accumulation of catecholamines by exchange for protons (103). Subsequently, it was demonstrated that a similar proton pump operates in the vacuoles of fungi and plants (23, 100, 195). Even though biochemical work identified the enzyme as the V-ATPase (38, 61, 184), it was the isolation of the yeast V-ATPase by Anraku and co-workers (207) that paved the way for detailed molecular biology studies of its structure and properties (150). The cloning of genes encoding V-ATPase subunits provided the first evidence that the F- and V-ATPases are related and have evolved from a common ancestor (21, 26, 122, 153, 233). These discoveries and others to follow reignited interest in a central problem in biology: how ATP and transmembrane voltages energize biomembranes for a wide spectrum of biological work.

In eukaryotic cells, F-ATPases are confined to the semi-autonomous organelles, chloroplasts, and mitochondria, which contain their own genes that encode some of the F-ATPase subunits (153). The F-ATPases are also present in the plasma membrane of every known eubacteria where they catalyze photosynthetic or respiratory ATP formation and generate pmf by catalyzing ATP-dependent proton pumping. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form ATP at the expense of the pmf, V-ATPases function exclusively as ATP-dependent proton pumps (153). Although we assume that a similar mechanism underlies ATP-dependent proton pumping by F- and V-ATPases in eukaryotic cells, the latter cannot catalyze pmf-driven ATP synthesis. The loss of this ability is probably due to alterations in the membrane sectors of this enzyme (153). The pmf generated by V-ATPases in organelles of eukaryotic cells is utilized as a driving force for numerous secondary transport processes. Although V-ATPases are complex in structure, they have but one primary function: to couple the hydrolysis of ATP to H^+ translocation across biological membranes. The cytoplasm is always rendered negative to the *trans*-side of the membrane because the protons carry positive charge away from the ATP-binding site, which, of course, always faces the cytoplasm. In most organelles, Cl^- is the gegenion for the transported H^+, and the vacuole becomes acidic.

Until recently, V-ATPase had been considered to be important in energizing plasma membranes of but a few specialized cells. The Na^+-motive force, produced by the Na^+-K^+-ATPase, was widely recognized to be the main plasma membrane energizer in animal cells (78). Animals use the Na^+-motive force to energize a set of Na^+-linked transporters, such as the Na^+-glucose cotransporter. It now appears that V-ATPases are as important as Na^+-K^+-ATPases for energizing animal plasma membranes (155, 219). The V-ATPase, usually localized in apical plasma membranes of epithelial cells, faces the cytoplasmic side of the membrane and pumps H^+ outward. Thus proton pumping always leads to a "*trans*"-positive voltage, but accompanying events may promptly dissipate it and the *trans*-compartment may become acidic, basic, or neutral, depending on the nature of parallel channels or porters and upon the counterion that accompanies the proton (80). For this reason, plasma membrane V-ATPases can energize the membrane, regulate intracellular pH, and bring about extracellular acidification or alkalinization.

In the past decade, impressive progress has been made in elucidating the properties, structure, and evolu-

tion of V-ATPases. The evolution of V-ATPases has been reviewed in several articles (56, 72, 151, 199). The structure, function, biogenesis, and regulation of V-ATPases have been recently reviewed (182), and two books were published on V-ATPase (82, 155). Recent reviews also emphasize plasma membrane V-ATPases and specialized functions in plants, microorganisms, and mammalian systems (12, 22, 46, 60, 70, 119, 156, 196, 219). However, we still do not know how V-ATPases couple ATP hydrolysis to H^+ pumping, nor do we understand how the many subunits of the membrane and catalytic sectors are assembled. We are just beginning to understand how the V-ATPase is assembled into the different membranes of a wide variety of organelles. We do not know how it is targeted to apical plasma membranes in some cells and to basolateral membranes in others. We do not understand how an enzyme as conserved as the V-ATPase can play such contrasting roles in specialized cells and organelles. Finally, we have no idea how such a complex enzyme could have appeared so soon after the origin of life or during its early evolution.

II. SUBUNIT STRUCTURE AND FUNCTION OF VACUOLAR PROTON-ADENOSINETRIPHOSPHATASES

A. Subunits Definition and Mechanism of Action

A typical SDS polyacrylamide gel of purified V-ATPase, in this case from bovine chromaffin granules, shows its characteristic subunit structure (Fig. 1). Although this preparation is what the biochemist calls "reasonably pure," biochemical methods always faced difficulties in the positive identification of a polypeptide as an integral subunit of a membrane protein. A subunit must be present in stoichiometric amounts and must be necessary for a complex activity as well as the assembly or stability of the holoenzyme. Purified biochemical preparations may contain irrelevant polypeptides or lack genuine subunits that were dissociated during the preparation of the complex without detectable effect on its assayed activity. The fact that V-ATPases are almost identical in their subunit composition in every eukaryotic cell enabled the utilization of yeast genetics for the determination of the subunit composition of the enzyme (see sect. II*B1*). Yeast genetics also allowed the identification of special properties of individual subunits and the discovery of factors that are involved in the enzyme biogenesis and assembly but not in its catalytic activity. The biochemical and genetic data are integrated in Figure 2 and Table 1 for the subunit structure and function of V-ATPases. We use the subunit nomenclature that was recently proposed (182). The widely accepted nomenclature of subunits A, B, C, D, E, F, and c (proteolipid) is retained (60, 82, 155, 156). Subunit G was originally named M16 or subunit b as the homolog of the

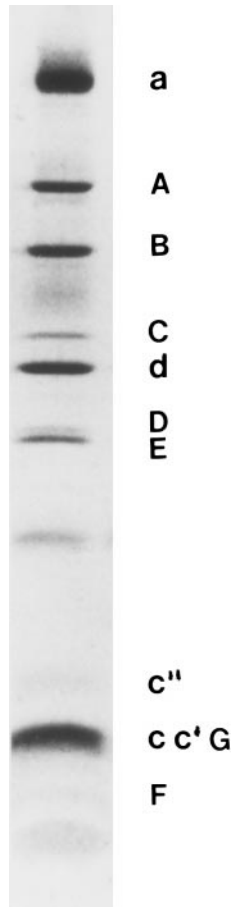

FIG. 1. Polypeptide composition of purified vacuolar H^+-ATPase (V-ATPase) from bovine chromaffin granules. Purified preparation was analyzed by electrophoresis on SDS polyacrylamide gel. Gel was stained with Coomassie blue. Subunit nomenclature was adopted from yeast enzyme (see Table 1). Mammalian subunit Ac45 appears as a diffused band between subunits B and C. Band between subunits E and c'' is a contamination by chromaffin granule membrane-cytochrome 561.

mitochondrial b subunit of F_o (156), subunit d was Ac39 or M39 (82, 156), subunit a was Ac115 or M115 (82, 156), and the additional proteolipids were denoted as c′ and c′′ (182 and Table 1).

The general structure of F- and V-ATPases is quite similar. Both holoenzymes are composed of catalytic sectors, F_1 and V_1, respectively, and membrane sectors, F_o and V_o, respectively. Recent advancement in our knowledge about the mechanism of action of F-ATPase (1, 98) has clarified structure-function relations of individual subunits of the enzyme. We will utilize the knowledge gained in the study of F-ATPase to assign each V-ATPase subunit to one of four parts of a mechanochemical machine: *1*) catalytic unit, *2*) shaft, *3*) hook, and *4*) turbine (Table 1 and Fig. 2). The assignment of individual V-ATPase subunits that have no homology with F-ATPase is based on scattered observations and therefore is rather speculative.

Definite functions have been assigned to but 4 of the

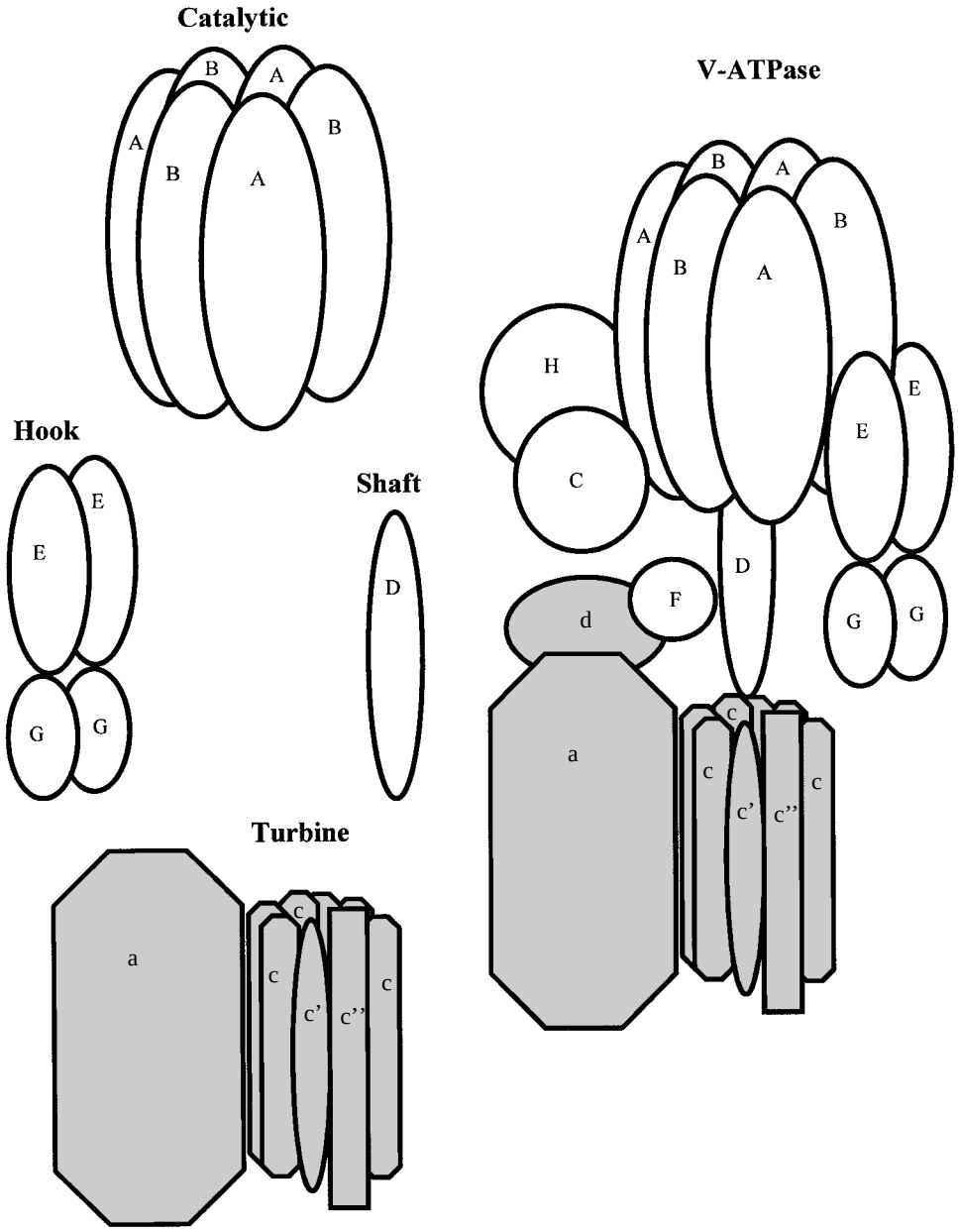

FIG. 2. Subunit structure of V-ATPase. Proposed organization of various subunits is based on studies described in text and assuming common general structure between F- and V-ATPases. Distribution of various subunits in 4 functional parts of enzyme is discussed in text. At least two copies of subunit G are present in each enzyme (192). A stoichiometry of 1 was reported for subunit E (182), but in most preparations, it appears to be more abundant than subunits C and D.

13 subunits that comprise the V-ATPase, the functional assignments being based primarily on homology to subunits in the F-ATPase (V-A to F-β, V-B to F-α, V-G to F-b, and V-c to F-c). It is assumed that the function of the homologous subunits is similar in F- and V-ATPases. However, b subunit of F-ATPase is a typical F_o subunit whereas the G subunit of V-ATPase was reported to be associated both with V_1 and V_o sectors (41, 112, 191, 192). The structure is most clearly conserved between V_1-A and F_1-β subunits and between V_1-B and F_1-α subunits. As noted above, the three-dimensional structure of the F_1 sector of F-ATPase from bovine heart mitochondria was recently determined at 2.8-Å resolution (1). The structure is asymmetric with a 40-Å stem that contains two α-helices in a coiled-coil arrangement. This stem presumably represents the γ-subunit that has been implicated in the mechanochemical mechanism for coupling of ATP hydrolysis to proton pumping and for coupling pmf to ATP formation. This tertiary structure and catalytic mechanism represent major advances in understanding energy coupling by ATP-dependent proton pumps that have far-reaching implications. The intriguing question is how much of the structure, and consequently the mechanism of action, will be preserved in V-ATPases? A bovine cDNA and a yeast gene (*VMA8*) encoding subunit D (Vma8p) of the respective V-ATPases were cloned (76, 147). Although no significant sequence homology was found between subunit D and the γ-subunit of F-ATPases, structural anal-

TABLE 1. *Subunit distribution and function of the yeast V-ATPase*

Subunit	Mass	Yeast Gene	F-ATPase Homolog	Proposed Function	Reference No.
			V_1 sector		
Catalytic					
A	68	*VMA1*	β	ATP hydrolysis	89
B	57	*VMA2*	α	ATP binding	145
Shaft					
D	28	*VMA8*		Analog of γ?	76, 147
Hook					
E	27	*VMA4*		Analog of δ?	62
G	13	*VMA10*	b	Hook	192
			V_o sector		
Turbine					
a	95	*VPH1*		Proton translocation?	124
	95	*STV1*		Proton translocation?	126
d	40	*VMA6*		Assembly?	17
c (proteolipid)	16	*VMA3*	c	Proton translocation	149, 209
c′	17	*VMA11*	c	Proton translocation?	208
c″	19	*VMA16*	c	Proton translocation?	4, 88
			Others		
F	14	*VMA7*		V_1-V_o assembly	75, 146
C	42	*VMA5*		V_1-V_o assembly	19
H	54	*VMA13*		ATPase regulation	93
	25	*VMA12*		Assembly, V_o in ER	90
	8.5	*VMA21*		Assembly, V_o in ER	86
	21	*VMA22*		Assembly, V_o in ER	87

ER, endoplasmic reticulum.

ysis indicated similar motifs in the two proteins. Similar analysis of subunit E (25, 62) led to the proposal that subunits D and E may both function in the V-ATPase stalk (205). Because the traditional stalk has now been divided into a shaft and a hook, we assigned subunit D to the shaft and subunit E to the hook (Fig. 2).

The main function of the catalytic sector is to bind ATP and to catalyze ATP hydrolysis. The main function of the membrane sector is to conduct protons across the membrane. The energy coupling between these two processes is believed to be catalyzed via mechanochemical-induced conformational changes in which the γ-subunit rotates as an axle within the α,β-complex (1). According to this concept, proposed by Paul Boyer two decades ago (for reviews, see Refs. 27 and 98), ATP hydrolysis catalyzed by subunits α and β of F-ATPase will induce a rotation moment to the "shaft" (subunit γ) that in turn will rotate the "turbine" (membrane sector subunits a and c) that pumps protons across the membrane. The "hook" (subunits b and δ) prevents the rotation of the catalytic unit. As we mentioned above, recent data with F-ATPases from bacteria and mitochondria support the function of the various subunits in this mechanochemical activity (98).

Which specific V-ATPase subunits should be assigned to which partial catalytic activity of the enzyme is not yet apparent. Figure 2 depicts the principal elements in a hypothetical mechanochemical V-ATPase machine. Subunits A and B of the catalytic sector are assumed to function in similar if not identical fashion to F-ATPase subunits β and α, respectively. Subunits E and G (Vma4p and Vma10p) are postulated to function as the hook and the proteolipids to function as the proton turbine. Subunit D (Vma8p) is the best candidate for acting as the rotated shaft (analogous to subunit γ of F-ATPase).

B. Structure and Function of Individual Subunits

1. Null mutations as criterion for subunit status

Null mutations in genes encoding V-ATPase subunits are likely to be lethal for most eukaryotic cells because primary energization of the vacuolar system by this enzyme drives vital secondary transport processes across membranes of vacuolar-derived organelles (153, 156). On the other hand, mutant *Saccharomyces cerevisiae* (yeast) cells presumably can survive the lack of acidification that results from disruption of genes encoding V-ATPase subunits by taking up acidic external fluid via endocytosis (143, 150). With the exception of *VPH1* and *STV1*, which encode homologous proteins (124, 126), all genes encoding subunits of the V-ATPase are present as a single copy in the yeast genome (153, 182). Disruption of each of the single-copy genes yields an identical phenotype in which cells cannot grow at a pH higher than 7 and are sensitive to low and high Ca^{2+} concentrations in the medium (150,

158, 209). The proteins that are present at stoichiometric amounts, and are encoded by genes whose mutations lead to this phenotype, must be valid subunits of the V-ATPase. Thus yeast genetics helped to sort out the genuine V-ATPase subunits and led to the discovery of some novel subunits as well as proteins that function exclusively in the assembly of the enzyme but are not genuine subunits. Disruption of genes encoding V-ATPase subunits in *Neurospora* and *Drosophila* is also lethal (24, 47). Null mutants of *Drosophila*, produced with the aid of P elements, have validated several V-ATPase subunits (47).

The subunit structure of V-ATPase is depicted in Table 1. It includes subunits that were shown by genetic means (see sect. IV) to be necessary for the function and/or assembly of V-ATPase in yeast and are present at significant amounts in the purified enzyme. Also included in Table 1 are some accessory gene products that are necessary for the assembly and/or function of the active enzyme but are not part of the final complex.

2. Catalytic subunits

A) SUBUNIT A. First cloned from plants and *Neurospora*, the 68-kDa subunit A (Vma1p) binds ATP and catalyzes its hydrolysis (26, 233). At the same time, a gene conferring trifluoperazine resistance was cloned from yeast cells and shown to be homologous to the β-subunit of F-ATPases (180). Only later was it recognized that this gene contains a nested genetic element and expresses two functional proteins (89). Vma1p is generated by a novel mechanism of protein splicing (102). The amino acid sequence of subunit A is >25% identical with that of F-ATPase subunit β. Like the β-subunit, subunit A contains a motif with the nucleotide-binding, glycine-rich, consensus sequence of GXXXXGKT/S that is common for ATP binding proteins (215). In contrast to the β-subunit, at this motif in subunit A there are two cysteine residues, one of which renders eukaryotic V-ATPases sensitive to -SH reagents (52, 136). Moreover, modification of a single cysteine of bovine subunit A prevents dissociation of the catalytic sector from the membrane by cold treatment (138). Presumably because of binding with these residues, *N*-ethylmaleimide (NEM) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) inhibit ATPase activity in an ATP-protectable manner. Both [^{3}H]NEM and [^{14}C]NBD-Cl, as well as 2-azido-[^{32}P]ATP and [^{32}P]ATP, label subunit A under suitable conditions (60). These and other observations leave little doubt that the ATP-binding site and ATP-hydrolyzing activity of the enzyme reside in the A subunit.

The similarity between subunit A of V-ATPases and the β-subunit of F-ATPases together with the resolution at 2.8 Å of the bovine F_1 has been a major factor in the selection of various regions for mutagenesis (121). Reversible disulfide bonding between C254 (bovine) of the glycine-rich motif and C532 suggested that they are involved in redox regulation of V-ATPase activity (53, 54). The distance between the corresponding residues in the β-subunit of F_1 is close enough to allow such a bond formation (1), suggesting that oxidation of these two cysteines lock the catalytic sector in a single inactive conformation. Modification of the same cysteine residue also prevented a dissociation of V_1 by cold inactivation, presumably by locking the enzyme in a conformation that prevented the cold-induced subunit dissociation (138). These and other results support the hypothesis that the A and β-subunits have similar folding patterns even though they have different molecular weights and their sequence similarity is not striking.

B) SUBUNIT B. First cloned from *Neurospora* and plants, the 57-kDa subunit B is homologous to the subunit α of F-ATPases (21, 123). In yeast, it is encoded by the *VMA2* gene whose disruption yields the phenotype that is typical for yeast cells lacking V-ATPase activity (145, 150). Although it exhibits greater sequence identity with F-ATPase α-subunit than subunit A with β, subunit B lacks the glycine-rich motif (215). Nevertheless, subunit B binds ATP analogs, but only under restricted conditions. Initial experimental support for an active ATP-binding site came from binding 3-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate (BzATP) to the plant subunit (125). Recently, it was shown that BzATP inhibits the bovine V-ATPase by modification of a single, rapidly exchangeable, noncatalytic binding site (212). These and other experiments suggest that subunit B functions as a regulatory subunit containing an ATP-binding site, and in addition, it may contribute important residues for the catalytic function of subunit A.

3. Shaft

A) SUBUNIT D. Subunit D was first cloned simultaneously from bovine adrenal medulla and yeast (147). The bovine and yeast proteins exhibit 55% amino acid sequence identity. Moreover, significant similarity was detected with subunits encoded by operons of two bacterial V-ATPase-like enzymes. Subunit D shows 20% identity with ATPG ("γ"-subunit) of *Sulfolubus acidocaldarius* V-ATPase (43) and 28% identity with the NtpD subunit of Na^{+}-ATPase of *Enterococcus hira* (200). In addition, similar structural motifs were identified in subunit D of V-ATPase and subunit γ of F-ATPases (147). These observations suggest that subunit D (*Vma8p*) may function in V-ATPase as the counterpart of the γ-subunit of F-ATPase. If indeed subunit D is the analog of subunit γ, it should play a crucial role in the coupling between ATP hydrolysis and proton pumping. With the assumption of similar mechanisms for F- and V-ATPases, subunit D will serve as the rotating shaft that gains its rotation moment from conformational changes in subunit A and delivers the torque to the proton turbine in the membrane sector.

4. Hook

A) SUBUNIT E. Subunit E was first cloned from V-ATPase isolated from bovine kidney (91). Subsequently, it was cloned from yeast (62) and designated as *VMA4* (92). Disruption of the yeast gene resulted in a phenotype identical to the other V-ATPase null mutants, and it was found to be necessary for V_1 assembly (44, 92). The predicted structure of E subunit contains elongated α-helices, which suggested that this subunit may be a part of the stalk (25). The bovine and yeast proteins are 34% identical and show no homology to F-ATPase subunits. Weak homology between subunit E and NtpE of Na^+-ATPase of *Enterococcus hira* was detected (200). It is unlikely that both subunits D and E function as a rotating shaft, and only structural data can reveal the identity of the subunit γ-analog. We propose that the functional status of subunit E is in the hook that was considered in the past to be part of the stalk. So far, the best evidence for this assignment comes from the observed interaction between subunits E and G in yeast (204).

B) SUBUNIT G. The gene (*VMA10*) and cDNA encoding this subunit in yeast and bovine preparations were cloned simultaneously (191, 192). Subsequently, it was cloned from insects and rats, suggesting that this subunit is a component of all V-ATPases (41, 112). Subunit G exhibits a significant homology to the b subunit of F-ATPases (191). Because subunit b is a constituent of F_o and functions as a hook that prevents rotation of the catalytic subunits, it was suggested that subunit G may have a similar function in V-ATPases (191). Cold inactivation left a significant amount of subunit G on the membrane (191, 192). However, in subsequent studies, this subunit was shown to be released by NO_3^- treatment, suggesting a V_1 association (112). This phenomena may result from the lack of a hydrophobic segment that is present in the b subunits of F-ATPases (41, 112, 191, 192). The hydrophilic part of the b subunit was expressed in *Escherichia coli* (48). The polypeptide formed a dimer whose sedimentation characteristic suggested an elongated shape and whose circular dichroism spectrum suggested a helical conformation. This evidence is consistent with structural predictions by several computation methods (214). Consequently, the b subunit of F_1 is viewed as having two long α-helices broken by a turn. Structural analysis of the yeast Vma10p indicates a structure almost identical to the b subunit (191); it has a very high tendency to form an α-helix and is broken in the middle by an apparent turn. These structural similarities suggest that subunit G of the V-ATPase, like subunit b of the F-ATPase, acts as part of the hook during the catalytic reaction.

5. Turbine

A) SUBUNIT C (PROTEOLIPID). The cDNA encoding a 16-kDa proteolipid (subunit c) was first cloned from bovine adrenal medullas (122). Subsequently, the gene encoding this V-ATPase subunit in yeast was cloned and interrupted, revealing the yeast phenotype of V-ATPase null mutants (149, 150, 209). In the F-ATPase, subunit c (proteolipid) is an 8-kDa protein containing ~80 amino acids. It is highly hydrophobic and soluble in a chloroform/methanol solution. The *E. coli* proteolipid has two transmembrane helices with a hairpin turn facing the catalytic sector in the cytoplasm. In the middle of the second helix, there is a glutamyl or aspartyl residue that provides the binding site for *N,N′*-dicyclohexylcarbodiimide (DCCD) (55, 66, 67). Binding of DCCD blocks proton conductance across the membrane and therefore inactivates the enzyme. In V-ATPases, subunit c (proteolipid) is also a highly hydrophobic protein that binds DCCD, but it contains ~160 amino acids and has a relative molecular mass of 16 kDa (149, 150). The DCCD binding inactivates the proton pumping and ATPase activities of the enzyme (6, 196). The sequences revealed that the proteolipid evolved by gene duplication and fusion of an 8 kDa-encoding ancestral gene homologous to that present in F-ATPases (122, 153). The sequence data also revealed four potential transmembrane helices with a single buried carboxyl group in helix IV that is thought to be the DCCD-binding site (158). The proteolipid is thought to be the principal subunit involved in proton translocation across the membrane.

B) SUBUNITS C′ AND C′′ (ADDITIONAL PROTEOLIPIDS). A short time after the first cDNA and gene encoding proteolipids in bovine and yeast were cloned, a second yeast gene encoding an homologous protein was cloned (4). This serendipitous cloning was neglected until Anraku and colleagues (88) discovered that in the yeast genome there exist three genes encoding proteolipids of V-ATPase: *VMA3* (the principal proteolipid), *VMA11*, and *VMA16* (88). Interruption of the genes encoding each of the additional proteolipids resulted in the typical V-ATPase phenotype. Moreover, substitution of the active glutamyl residue (to Ala or Gly) in each of the proteolipids inactivated the V-ATPase but produced a fully assembled enzyme that was localized in the vacuole (88, 158). These data suggest that the yeast V-ATPase contains at least one copy of subunits c, c′, and c′′ and that each of them is essential for the activity of the enzyme. Recently, the genes encoding subunits c′ and c′′ were cloned from *Caenorhabditis elegans* (161). Plant and mammalian expressed sequence tags (est) encoding subunit c′′ are present in the GenBank, and it is likely that c′ will also be a constituent of other V-ATPases.

Why is one proteolipid sufficient for F-ATPases, whereas three of them are required for V-ATPases? The likely mechanistic constraint that may have led to the evolution of this deviation is the change in the coupling efficiency of V-ATPases that is referred to as the "slip" mechanism (137, 153; see below).

C) SUBUNIT A. The cDNA encoding this subunit of ~100 kDa was first cloned from rat brain (168). Subunit a is composed of an NH_2-terminal hydrophilic domain of ~45 kDa and a COOH-terminal hydrophobic domain of ~55 kDa, with six or seven putative transmembrane segments. The hydrophilic globular domain is facing the cytoplasmic side of the membrane (94). Subunit a is glycosylated in mammalian V-ATPases, but the site of glycosylation is not known (5). Only a single gene encoding this subunit was identified in mammalian cells, although splicing variations have been reported (167). In yeast, subunit a is encoded by two genes that were named *VPH1* and *STV1* (124, 126). In contrast to all the other V-ATPase subunits, null mutation in each of the two genes could grow at high pH and with high or low Ca^{2+} concentrations in the medium. However, disruption of both genes resulted in a phenotype similar to all the other V-ATPase null mutations (126). This observation suggests that one copy of subunit a is necessary for the activity and/or assembly of the enzyme. The discovery of this null phenotype made it possible to study amino acid replacements by site-directed mutagenesis (111). Some amino acid changes in the last putative transmembrane domains of Vph1p, in particular E789Q, reduced the proton pumping activity without affecting the assembly of the enzyme. The results were similar to changes in *E. coli* F_o subunit a (35), and it was proposed that Vph1p is the analog of subunit a of F-ATPases (111). Because there is no homolog of subunit a encoded by the yeast genome, this argument may be acceptable, and we also adopted the nomenclature of subunit a for the former Ac115 (153, 182). The two proteins Vph1p and Stv1p are ~50% identical and may function in V-ATPases of different cellular organelles. Thus Stv1p is proposed to replace Vphlp in the Golgi-resident V-ATPase (126). The assignment of this V-ATPase subunit as an analog of F-ATPase subunit a is still in the hands of the jury.

D) SUBUNIT d. Subunit d was initially cloned from bovine adrenal medulla and denoted as an accessory V-ATPase subunit AC39 (216). Subunit d is also unique to V-ATPases, where it is associated with the membrane sector but contains no apparent transmembrane segments (216). It is predicted to interact with vacuolar membranes by direct interaction with other V_o polypeptides. The yeast gene encoding this subunit (*VMA6*) was cloned and interrupted, yielding the typical V-ATPase null phenotype (17). Extraction of subunit d by alkaline carbonate indicated that it is peripherally associated with the V_o sector on the cytosolic side of the membrane. In null mutants for V_1 subunits, subunit d assembles to the V_o sector, and the assembled complex is targeted to the vacuole (17). In mutants in which *VMA6* was disrupted, V_1 fails to associate with V_o, suggesting a possible function in the association of the two parts of the enzyme.

6. Other subunits

A) SUBUNIT C. This subunit was first cloned from bovine adrenal medulla by obtaining amino acid sequences from the purified subunit of the bovine adrenal medulla enzyme (148). Subsequently, the gene *VMA5* encoding this subunit in yeast was cloned and interrupted (19), resulting in the typical V-ATPase null phenotype. Vma5p was found to be necessary for the assembly of complete V-ATPase (19, 92) but not for its V_1 part (44). The two proteins from bovine preparations and yeast are 37% identical, and like all other V_1 subunits, subunit C is released from the membrane by cold inactivation (19). Subunit C exhibits no homology to any of the F-ATPase subunits, and consequently, its function and distribution in the four functional parts of the enzyme are not known. Subunit C is not necessary for the assembly of the other V_1 subunits in bovine coated vesicles (172). However, Ca^{2+}-dependent ATPase activity was not observed in V_1 complexes that lack this subunit (167a). On the other hand, a V_1 from *Manduca* exhibited Ca^{2+}-dependent ATPase activity without subunit C (73). Therefore, its classification as a catalytic subunit or a regulatory one should await further experiments.

B) SUBUNIT F. Subunit F was first cloned from caterpillar midgut (74) and subsequently from yeast (75, 146). Disruption of the gene *VMA7* encoding subunit F in yeast resulted in a typical phenotype of V-ATPase null mutants. Unlike other V_1 subunits in yeast, the absence of subunit F disrupts not only the assembly of V_1 but also the assembly of V_o (75). Its hydropathy plot and release by cold inactivation show that subunit F is a V_1 subunit. Bovine cDNA encoding this subunit was cloned and a recombinant protein stimulated ATPase activity of reconstituted V_1 (226). Both ATP hydrolysis and proton transport are inhibited by a monospecific antibody to the insect subunit F (74). A monoclonal antibody against hemagglutinin (HA) tag inhibited proton pumping activity of yeast vacuoles having V-ATPase with a HA-tagged subunit F (146). Because it is present in such distantly related organisms, subunit F is thought to be a universal subunit. The inhibition of ATPase activity by subunit F specific antibodies and its solubility properties may suggest that this subunit is an analog of the F-ATPase δ-subunit. Therefore, we tentatively assign it to the hook.

C) SUBUNIT H. Preparations of purified V-ATPase from various sources exhibited some polypeptides that migrated below subunit B on SDS gels (68, 70, 166). The identity of these polypeptides as genuine subunits of the enzyme was debatable until the yeast *VMA13* gene was cloned and interrupted (93). The phenotype of the null mutant was similar to other V-ATPase null mutants except that all the other subunits were assembled into a full complex, with no ATPase or proton-pumping activity (93). Recently, a homolog of the yeast Vma13p was identified in the clathrin-coated vesicle V-ATPase, suggesting

that this polypeptide is widespread and may be considered as subunit H of the enzyme (226, 232). This subunit is likely to function in the regulation of the enzyme activity and communication between V-ATPase and proteins connected with other cellular processes. Full recognition as a genuine subunit of V-ATPase will be gained only if the yeast null mutant fails to be suppressed without the expression of an extrinsic protein.

D) AC45 PROTEIN. The membrane of chromaffin granules contains several glycoproteins, some of which have had functions ascribed to them (225). One of them, glycoprotein IV, was found in association with the membrane sector V_o of V-ATPase (63, 187). The gene encoding this protein (Ac45) was cloned and shown to contain multiple potential glycosylation sites in accord with its migration on SDS gels as a diffused band (187). The cDNA encodes a 50-kDa protein that contains a membrane translocation signal at its NH_2 terminus. Expression of the cDNA in the presence of pancreatic microsomes yielded a glycosylated product (187). Enzymatic deglycosylation of the purified protein from chromaffin granules yielded a protein with an apparent molecular mass of 29 kDa (63, 187). It was suggested that in chromaffin granules additional NH_2-terminal processing takes place (63). There is no counterpart of Ac45 in the yeast V-ATPase, and the protein is not present in all the mammalian V-ATPases. The lack of yeast genetics as a tool makes the convincing assignment of Ac45 as a V-ATPase subunit quite difficult. A possible function for Ac45 as an organelle and membrane specific targeting protein was proposed (187).

III. BIOGENESIS AND ASSEMBLY OF VACUOLAR PROTON-ADENOSINETRIPHOSPHATASES

The assembly of the intact catalytic sector in yeast cells is dependent on the preassembly of the membrane sector; inactivation of each of the genes encoding integral membrane subunits of this enzyme prevents functional assembly (44, 150, 158). Therefore, the key to the differential assembly of V-ATPase in different organelles of eukaryotic cells lies primarily in subunits of the membrane sector. Because the subunits that comprise the membrane domain contain no cleavable signal sequence or propeptide, the signal for the assembly of each subunit appears to reside within the mature subunit. Specific receptors in the endoplasmic reticulum (ER) and/or Golgi may guide V-ATPases into specific organelles where they may be retained and fulfill secondary functions. On the other hand, the well-known membrane flow between organellar and plasma membrane (e.g., during endocytosis/exocytosis cycles) suggests that a dynamic exchange of V-ATPase may occur between endomembranes and plasma membrane (83).

Yeast cells have been the primary objects for the study of V-ATPase assembly (156, 182). Both plant and animal systems are complicated because tissue-specific or developmental stage-specific isoforms of the V-ATPase subunits exist (70, 196). Fortunately, in yeast, all subunits of the V-ATPase are encoded by single genes (with the exception of *VPH1/STV1* as noted above). The yeast system is therefore ideal for carrying out an organized and systematic mutational analysis to determine the properties of individual ATPase subunits and their contribution to the intact enzyme complex. All of the genes encoding structural proteins of the V-ATPase have been cloned, sequenced, and disrupted (156, 182). Initial studies with null mutants showed very clearly that each of the V-ATPase subunits is required for the proper assembly of the holoenzyme (see Refs. 17, 19). In general, all of the V_o subunits are required for assembly of the V_1 sector onto the membrane. The proteolipid (Vma3p) plays a central role in V-ATPase assembly, since none of the remaining V_o subunits assembles in its absence (17, 44, 150). The only exception is subunit F (Vma7p), which is considered to be a V_1 constituent, but null mutation in its gene disrupts not only the assembly of V_1 but also the assembly of V_o (75).

Partial complexes of V_1 subunits can form in the cytoplasm in the absence of an assembled V_o domain (44, 205). With the use of a native gel electrophoresis system, which allows a fine resolution of cytosolic V-ATPase complexes, a major cytosolic V_1 complex (complex II 576 kDa) was detected in wild-type yeast as well as in Δ*vma3* and Δ*vma5* strains (see Fig. 3). Strains having mutations in genes encoding the Vma1p, -2p, -4p, -7p, or -8p V_1 subunits fail to assemble this complex, although large, intermediate-sized complexes were sometimes detected. For example, a 317-kDa complex containing Vma1p, -2p, -7p, and -8p in the Δ*vma4* strain was observed (complex IV; Fig. 3). A 96-kDa complex (complex III) that appears to contain Vma4p and Vma10p in all strains except the Δ*vma4* and Δ*vma10* strains was also demonstrated (203). Subunit E (Vma4p) is required for assembly of V_1 onto V_o and for the interaction of Vma1p and Vma2p with other peripheral subunits (93, 204). Figure 3 depicts a schematic presentation of the various steps and subcomplexes that have been identified by null mutation to be part of the V-ATPase assembly and targeting mechanism. This scheme was adapted from Figure 9 in a recent paper from the laboratory of Klionsky et al. (204).

Several newly discovered genes encode proteins that are not a part of the V_o complex but that affect its assembly (86, 90, 204). Vma21p is a particularly interesting integral membrane protein of 9.5 kDa (86) that resides in the ER where it is required for the assembly of the V_o sector. Moreover, the unassembled Vph1p is rapidly degraded in the mutant lacking Vma21p. A homologous

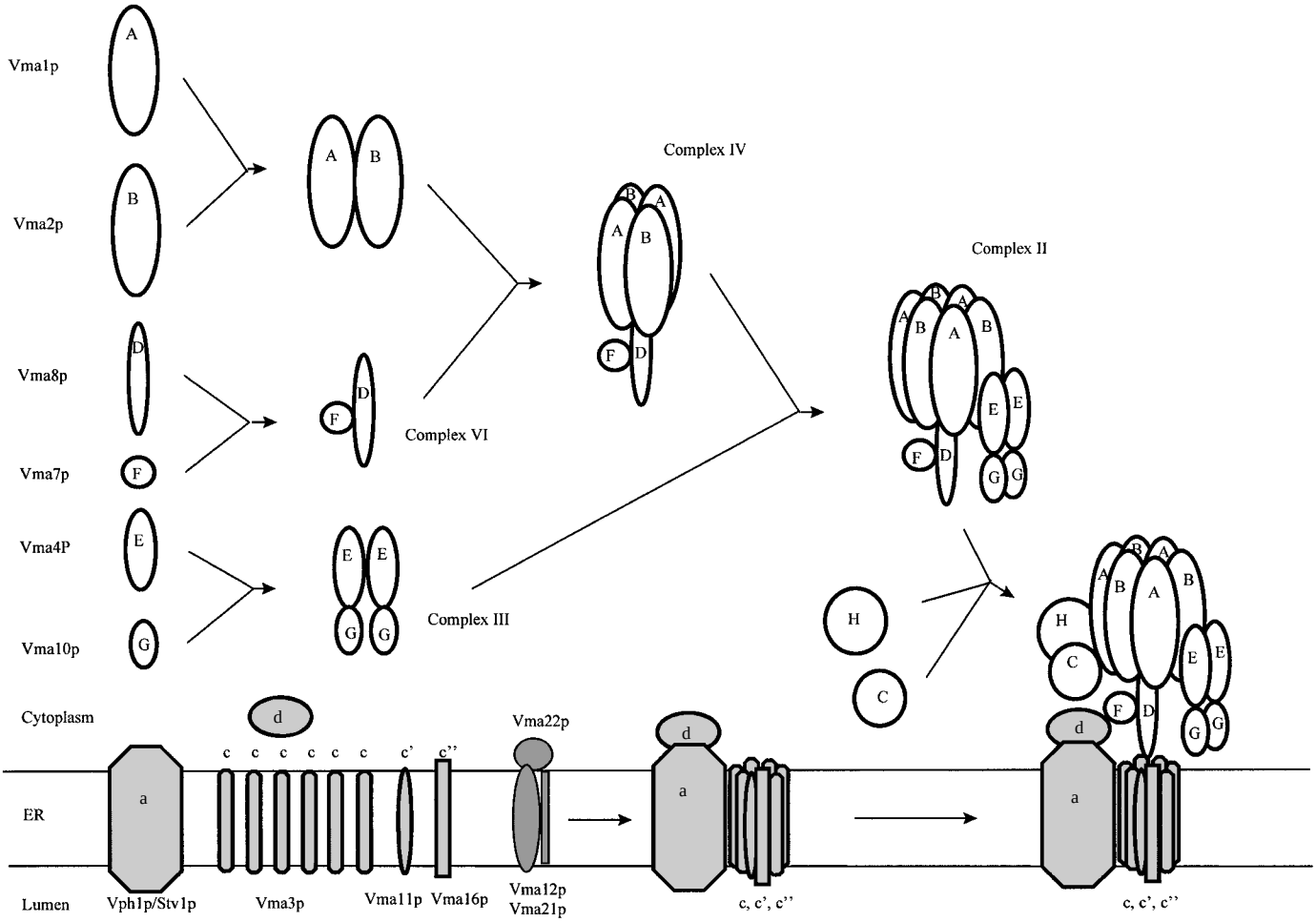

FIG. 3. Steps in assembly of yeast V-ATPase. Scheme was drawn from Figure 9 of Reference 204, and various steps were modified to accommodate general structure depicted in Figure 2. Subcomplexes are described in text and in Reference 204. [Modified from Tomashek et al. (204).]

protein to Vma21p was recently identified in V-ATPase from chromaffin granules (118). The 21-kDa Vma22 protein is also an ER-localized protein required for V-ATPase assembly (204). As with Vma21p, the absence of Vma22p results in degradation of Vph1p and prevents the assembly of V_1 onto the membrane. The association of Vma22p with the ER is itself dependent on another assembly factor Vma12p (25 kDa). Again, mutants defective in Vma12p have low levels of V_o subunits in the vacuole membrane and fail to assemble the peripheral sector onto the membrane (90, 204). These results suggest that V_o assembles in the ER and is subsequently moved to other locations within the cell. In the absence of correct assembly, the integral membrane domain may be degraded at the level of the ER. These studies are important first steps toward understanding how the membrane sector is assembled and targeted to the correct locations within the yeast cell.

IV. MOLECULAR GENETICS OF VACUOLAR PROTON-ADENOSINETRIPHOSPHATASES

As described in section II, more than 15 yeast genes encode proteins that are involved in the structure and/or biogenesis of V-ATPases. These gene products fall into three main categories: *1*) proteins that are directly assembled in stoichiometric amounts into the V-ATPase holoenzyme, *2*) proteins that are involved in the biogenesis and assembly of V-ATPases but are not genuine subunits, and *3*) regulatory proteins that interact with the enzyme.

Yeast cells provide a special opportunity to analyze V-ATPase subunits by site-directed mutagenesis. Because all genes that encode subunits of the V-ATPase (except *VPH1* and *STV1*) are present as a single copy in the yeast genome, their disruption yields an identical phenotype in which cells cannot grow at a pH higher than 7 and are sensitive to low or high Ca^{2+} concentrations in the me-

dium (150). Not only does this phenotype make possible the analysis of subunits by site-directed or random mutagenesis but also it enables the production of second-site suppressors for inactive mutations (158, 190). To determine the function of mutated genes, the interrupted mutants are transformed with plasmids containing intact or modified genes, and growth at pH 7.5 indicates the presence of a functional gene.

A. Mutations in the Catalytic Sector

Results from mutations of F-ATPase guided mutational analysis of the catalytic subunits of V-ATPases. The experiments were performed mainly in yeast because of the unique phenotype of the null mutations in individual subunits (150). Two principal locations were addressed: the cysteine residues and the area corresponding to residues observed in the F_1 crystal structure to be in proximity to the adenine ring of bound ATP (114, 116, 121, 198). The A subunit of the yeast V-ATPase contains three highly conserved cysteines: C261, C284, and C539. Of the three cysteine residues, two (C284 and C539) appear essential for correct folding or stability of the A subunit. Mutation of the third cysteine (C261), located in the glycine-rich loop, to valine, generated an enzyme that was fully active but resistant to inhibition by NEM, NBD-Cl, and oxidation. This result supports the notion that C261 is the site of inhibition by NEM and other cysteine-modifying agents as well as in vivo oxidative inactivation of the enzyme (162). Changing either C284 or C538 to serine prevented growth at pH 7.5, suggesting that these residues are essential either for assembly or catalysis.

Mutation of G250D in the glycine-rich loop also resulted in destabilization of the A subunit, whereas the mutation K263Q gave a V-ATPase complex that showed normal levels of A subunit on the vacuolar membrane but was unstable and was totally lacking V-ATPase activity (114). Mutation of the acidic residue, which has been postulated to play a direct catalytic role in the homologous F-ATPases (E286Q), had no effect on stability or assembly of the V-ATPase complex but also led to loss of activity (116). The gene encoding this subunit A was also randomly mutagenized (114). The mutations S811P and E740D resulted in enzymes that assembled fully but were incapable of ATP hydrolysis. Conservative substitutions in F452, Y532, and F538, which according to the F_1 crystal structure should be close to the bound ATP, had little or no effect on V-ATPase activity (121). However, the mutant F452A had no activity. These results stress the limited information that can be gained by site-directed mutagenesis without the utilization of second-site suppressors.

Recently, site-directed mutagenesis was used to analyze the nucleotide binding sites on subunit B (Vma2p) in the V_1 domain (115). The mutation R381S eliminates proton pumping activity and dramatically reduces ATPase activity. This residue is predicted to contribute to the catalytic nucleotide-binding site present on subunit A (Vma1p). Glycerol gradient fractionation was used to assess assembly and/or stability of several V_1 complexes that resulted from the directed mutations. A partial instability of the peripheral domain resulting from the R381S mutation suggests that R381 may form part of a contact site with the A subunit. Random mutagenesis of the *VMA2* gene revealed that two mutations, E317K and E355K, resulted in loss of interactions between Vma1p and Vma2p (J. Tomashek and D. Klionsky, unpublished data).

B. Mutation of the Yeast Proteolipids (c, c′, c′′), Vma3p, Vma11p, and Vma16p

The proteolipid (Vma3p) was the first V-ATPase subunit to be studied by site-directed mutagenesis (158), because of its small size and because it is one of the most conserved hydrophobic proteins in nature (149, 153). Only 24 of 65 substitutions resulted in inactive proteolipids that could not support growth at pH 7.5. Second-site suppressors were generated for some of the inactive mutations (190). One of the inactive mutants, Q90K, was chemically mutagenized and screened for suppressors (190). All second-site mutations, including I89L or M, V74I, AI4V, and Q90N, that suppressed the Q90K mutation were intragenic. All amino acids that suppressed the mutation Q90K were either larger in size or had a changed topology of the side chain when compared with the original residue. These results suggest that suppression of the original mutation exhibits space-filling properties and that the residues A14, V74, I89, and Q90 are located close to each other in a tightly packed structure, although long-distance conformational charges could not be excluded.

The mutagenesis of the proteolipid also pointed to residue E137 as the DCCD-binding site and suggested that it may be involved in proton conductance. Substitutions of E137 and neighboring residues (158, 190) again revealed that second-site mutations which restore function are replacements of larger amino acids by smaller ones, thus compensating for the original change in the inactive mutant. Even though these results cannot be interpreted in simple mechanistic terms, the character of the changes indicated potential contact between helices II and IV. However, a model, derived from high-resolution electron microscopy of an isolated proteolipid from *Nephrops norvegicus* (96), suggested that there is intermolecular interaction between helices II and IV. Apparently, one cannot yet differentiate between inter- and intramolecular interactions between the helices of the proteolipid. Cysteine-scanning mutagenesis of *Nephrops norvegicus* subunit c, expressed in yeast proteolipid null mutant, showed that helix I residues sensitive to cysteine replacements were

clustered on a single face of the transmembrane segment (97). The proteolipid of these mutants was inaccessible to fluorescein 5-maleimide, and only residues that were insensitive to the mutation were modified by the reagent. It was suggested that the fluorescein maleimide-accessible face of helix I lines a pore at the center of a hexameric complex formed by the proteolipid (97). From mutation of the hydrophobic core of the chymotrypsin inhibitor and interactions of α-helices in a model system it was concluded that subtle changes in the size of the amino acid residues at the interface of two helices can cause significant destabilization (110). The site-directed mutagenesis and suppressor mutations in the yeast proteolipid support this conclusion (158).

Helix III of the proteolipid is rich in glycines that are conserved in all proteolipids of eukaryotic cells (154). These glycines are located at one face of the helix and therefore may play a role in the assembly of the membrane sector or even in its mechanism of action. The replacement of these glycines by valines in each of five cases inactivated the enzyme (190). In contrast, the overall inactivation rate in the previous extensive mutagenesis was ~35% (158). Apparently, this face of helix III is very sensitive to volume and/or hydrophobicity changes of its amino acid residues. This sensitivity may be due to tight structural constraints inside each proteolipid monomer, or it may indicate that this face of helix III is important for the formation of the proteolipid oligomers (96). Replacement of these glycine residues was much more deleterious than substitutions in helix IV in the vicinity of E137. A suppressor mutant for the substitution G101V was isolated and identified as a second-site substitution of I134V in helix IV. This isoleucine residue is situated in close proximity to E137. This suppression provides additional support for the notion that the size of the amino acid residues and not their hydrophobicity is the determining factor in the inactivation of the proteolipid and in the suppression of the inactive mutations. In contrast to the mutation G101V, the mutations G98V and G1O5V were not suppressed even after extensive mutagenesis of the proteolipid. This failure may suggest that these two glycines are either necessary for the activity of the proteolipid or necessary for specific interaction with another subunit.

The two additional proteolipids (subunits c′ and c′′), which were discovered in the yeast V-ATPase, present a very challenging mechanistic problem. Mutational analysis of these subunits revealed that both proteins contain a glutamic acid residue (c′ E145 and c′′ E108) that is functionally similar to subunit c E137 (88). These residues could be functionally substituted by an aspartic acid residue resulting in a V-ATPase that exhibits only ~5% of the original activity in subunit c (114, 158) and fully active enzyme in subunits c′ and c′′ (88). Other substitutions of E137Q, E137V, and E137K in subunit c (158) or E145L and E145Q in subunit c′ and E108L, E108Q, and E108V in c′′ completely eliminated the V-ATPase activity (88). However, only subunit c′ E145Q or subunit c′′ E108Q, but not subunit c E137Q mutations, encoded by a low copy plasmid on wild-type background were able to grow at pH 7.5. This finding, together with the observation that subunit c is the most abundant form, suggests that subunit c is the major proteolipid subunit in the complex (88). Labeling by DCCD of V-ATPases from chromaffin granules and coated vesicles yielded a very strong band at 16 kDa and a band with lower intensity at ~19 kDa (7, 140). This second band probably reflects the presence of subunit c′′ in the mammalian enzyme. This subunit is present in *C. elegans*, and cDNA fragments capable of encoding parts of subunit c′′ have been isolated from plants and mammalian cells (161). In summary, V-ATPases appear to contain three proteolipids; one of them is the principal proteolipid (subunit c) that is present in six copies per enzyme (Fig. 2). The other two may be present at one copy per enzyme and function in activities unique to V-ATPases of eukaryotic cells. The presence of the additional proteolipids and the duplication of the principal proteolipid from two to four transmembrane segments give the eukaryotic V-ATPases their unique properties.

V. CELL BIOLOGY OF YEAST VACUOLAR PROTON-ADENOSINETRIPHOSPHATASE NULL MUTANTS

Acidic pH inside the vacuolar system of eukaryotic cells is necessary for the function of numerous vital cellular processes (129, 131). Consequently, most eukaryotic cells are unlikely to survive mutations that inactivate their V-ATPases (153). So far, only *Saccharomyces cerevisiae* has been found to survive mutations that inactivate the enzyme. The phenotype of these mutants that cannot grow at high pH or high and low Ca^{2+} concentrations suggested that Ca^{2+} homeostasis may be the critical factor involved in both the lethality of all the tested eukaryotic cells as well as the survival of *Saccharomyces cerevisiae* at low pH (150, 158, 160). Vacuolar H^+-ATPase null mutations are pleiotropic and affect several other cellular processes. A pet^- phenotype that cannot grow on a nonfermentable carbon source is one of the consequences of V-ATPase null mutations (160). Later on it was demonstrated that the concentration of the nonfermentable carbon source is critical for the vitality of the mutants, and at low concentrations their growth is normal (190). Sorting of secretory proteins in the Golgi is also affected by the lack of V-ATPase activity (107, 227). Strains with chromosomal disruptions of the genes encoding the A, B, and c subunits of the V-ATPase accumulate precursor forms of the vacuolar membrane protein, alkaline phosphatase, and the soluble vacuolar hydrolases carboxypeptidase Y and proteinase A (228). The intracel-

lular precursors in V-ATPase null strains accumulate within the secretory pathway at some point before delivery to the vacuole but after transit to the Golgi complex. Vacuolar hydrolase-invertase hybrid proteins are inefficiently delivered to the vacuole in the V-ATPase null strains as demonstrated by vacuole isolation. Subcellular fractionation indicates that significant amounts of the carboxypeptidase Y-invertase and alkaline phosphatase-invertase hybrid proteins are located in the late Golgi complex and/or post-Golgi compartments (228). Thus V-ATPases are intimately involved in the secretory pathway, but their absence does not shut down the system; therefore, the lack of growth in their absence could not be attributed to an interference with biogenesis processes in the pathway.

Vacuolar H^+-ATPase is also intimately involved in endocytosis and receptor recycling (119, 129). This involvement may explain the sensitivity of V-ATPase null mutants to low metal ions in the medium (10, 188, 189). Here too, the absence of V-ATPase activity cannot explain the cause of lethality in eukaryotic cells with inactive enzyme. On the contrary, fluid-phase endocytosis is necessary for the survival of V-ATPase null mutants (143). Therefore, the critical effect that results in lack of growth at high pH of yeast null mutants has not been identified yet. Recently, it was reported that the growth inhibition caused by concanamycin A, a specific inhibitor of V-ATPases, of *Neurospora crassa* could be suppressed by mutations in the *PMA1* gene encoding the plasma membrane P-type H^+-ATPase (24). The mutations may affect the distribution of the enzyme and expression in organelles in which V-ATPase has a critical role; that way, missorting of the plasma membrane proton pump led to the replacement of V-ATPase that normally functions in these organelles. In looking for suppressors for inactive V-ATPase subunits, we discovered several recessive mutations that cause growth of V-ATPase null mutants at pH 7.5 (L. Supekova, F. Supek, H. Nelson, and N. Nelson, unpublished data). These yeast strains were obtained by ethyl methanesulfonate (EMS) mutagenesis of yeast with deleted genes encoding V-ATPase subunits. Consequently, the V-ATPase is totally inactive in these suppressor mutants. The mutagenesis caused inactivation of a protein complex, and it resulted from independent mutations in several complementation groups (169). Isolated vacuoles from these mutants did not show any ATP-dependent proton pumping activity, and intact cells fail to accumulate quinacrine into their vacuoles. Recently, we identified a family of novel genes (*VTC1–4*) that disruption of some of them induces growth of V-ATPase null mutants at pH 7.5 (A. Cohen, N. Perzov, H. Nelson, and N. Nelson, unpublished data). These genes encode membrane chaperons that influence the distribution of membrane proteins including V-ATPase. Resolving the mechanism by which their absence suppresses the lack of growth of V-ATPase null mutants at high pH may shed light on its function in key biogenesis processes.

VI. VACUOLAR PROTON-ADENOSINETRIPHOSPHATASES AS PLASMA MEMBRANE ENERGIZERS

Vacuolar H^+-ATPases play a major role as energizers of animal plasma membranes, especially apical plasma membranes of epithelial cells (see review in Ref. 219). This role was first recognized in plasma membranes of lepidopteran midgut and vertebrate kidney as well as in phagocytic cells and osteoclasts, but V-ATPases are now known to energize plasma membranes in classical models of ion transport, such as the frog skin (79, 83), and new roles are emerging, such as maintaining an acid environment for sperm maturation in mammalian epididymis (28, 32). The list of animals with plasma membranes that are energized by V-ATPases now includes members of most, if not all, animal phyla. A balanced view of membrane bioenergetics would recognize H^+ and Na^+ as the primary energizers of biomembranes, with Ca^{2+} and K^+ being close seconds, and with other ions being important in special roles. This view has long been accepted for bacteria, fungi, plants, and animal endomembranes. What is new is the realization that it probably applies to many animal plasma membranes as well.

In insects, a wide variety of processes, ranging from fluid secretion and absorption through alkalinization of extracellular fluids to sensory signal generation, are energized by plasma membrane V-ATPases (83, 80, 219). Processes such as nutrient uptake, which are energized by P-type Na^+-K^+-ATPases in mammals, utilize V-ATPases as a primary energy source for driving secondary uptake processes in many insects. The use of H^+ rather than Na^+ may be due to the low levels of Na^+ in the plant diet (80, 83). Freshwater animals ranging from clams to frogs are now known to take up Na^+ from dilute ponds and streams by processes that are secondary to primary V-ATPase activity.

Why would V-ATPase have been selected during evolution to energize plasma membranes alongside with Na^+-K^+-ATPase? One explanation may be the presence of plant alkaloids in the insect's diet. Some of those alkaloids are strong inhibitors of P-type ATPases such as the Na^+-K^+-ATPase. The low Na^+ concentration in fresh water makes Na^+ uptake down a chemical gradient generated by a basolateral Na^+-K^+-ATPase difficult. Uptake of Na^+ from fresh water is energized by an electrogenic V-ATPase in frogs, fish, crabs, and clams (163). It seems likely that similar energization of Na^+ uptake by V-ATPase in freshwater organisms will turn out to be a general phenomenon.

A. Lepidopteran Midgut Epithelium Energized by a V-ATPase

The lepidopteran midgut is composed of a single epithelial cell layer with a basal lamina and thin muscle covering (2, 39). Sodium concentrations are low in cell, lumen, and blood, and K^+ is the principal extracellular as well as intracellular cation, reflecting the plant diet. The alkalinity of the lepidopteran larval midgut (pH 10–11) is achieved by a V-ATPase accompanied by a $K^+/2H^+$ antiporter. The pump is located in the apical membrane of the goblet cells, one of the two principal cell types of the midgut epithelium (2, 39). Because the midgut lacks a Na^+-K^+-ATPase, all solute fluxes, including the absorption of amino acids and the regulation of the high pH in the midgut lumen, appear to be energized by the K^+ pump, which consumes ~10% of the larva's total ATP production (45, 46, 65). Highly purified goblet cell plasma membranes were isolated by utilizing their freedom from mitochondria (81). Unlike F_1 sectors of F-ATPases, which can be visualized only by special techniques such as negative staining, the large V_1 sectors of V-ATPases are readily observable in high-resolution electron micrographs. V_1 sectors (designated "portasomes") served as markers for the isolation of clean goblet cell apical membranes (GCAM) (40, 81). The subsequent identification, solubilization, and purification of V-ATPase from this fraction provided clear biochemical confirmation that the enzyme is a V-ATPase and is localized to this precise plasma membrane sector in the complex midgut epithelium (178, 218, 220–222). In contrast, it is impossible to distinguish between endomembrane and plasma membrane V-ATPases on the basis of cloning results. The cDNA encoding V-ATPase subunits are presumably present in all eukaryotic tissues. Immunolabeling with antibodies to V-ATPase subunits can localize the enzyme to an approximate sector of a cell. However, electron micrographs of portasome-studded plasma membranes, such as those in midgut GCAM, intercalated kidney cells, and ion-transporting cells of sensory sensilla can refine the localization of the V-ATPase to the plasma membrane (83).

Although the V-ATPase pumps H^+ from the intracellular to the extracellular side of the goblet cell apical membrane, the luminal pH is alkaline rather than acidic (45, 46). Activity of the V-ATPase hyperpolarizes the membrane to >240 mV (46). An electrophoretic $K^+/2H^+$ antiporter with an H^+:K^+ stoichiometry >1 (9, 220) drives H^+ from the midgut lumen to the cell and drives K^+ from the cell to the midgut lumen, resulting in net K^+ secretion and thus alkalinization of the midgut lumen (218).

The midgut transepithelial voltage is abolished during molting. Simultaneously, ATP hydrolysis and ATP-dependent proton transport are reduced drastically by the dissociation of the peripheral V_1 subunits from the membrane V_o complex (132, 186). The V_1 complexes appear to dissociate as a whole, since the cytosolic concentration of V_1 ATPase is doubled during the molt (73). After the molt, cytosolic V_1 complexes reassociate with the membrane V_o complexes, in the absence of protein biosynthesis. Reversible dissociation of the V_1V_o holoenzyme has also been observed during starvation, indicating that, as during molt, the disassembly may be a response to a drop in energy utilization by ion transport processes. Regulation of V-ATPase activity by dissociation and reassembly of V_1 and V_o complexes has also been reported in yeast (101, 165). When yeast are grown without glucose, V_1 and V_o complexes dissociate rapidly, only to reassemble when glucose is restored, probably due to a drop in intracellular pH. These similar findings from two phylogenetically distant organisms may indicate that dissociation and reassociation of their V_1 and V_o complexes may be a general mechanism for V-ATPase regulation.

B. Frog Skin Utilizes V-ATPase for Na^+ Absorption

Frogs, like most freshwater animals, absorb Na^+ and Cl^- through their skin from dilute pond water (49–51, 83, 106). The short-circuit current across isolated frog skin in Ringer solution is carried by Na^+; the mucosal (pond side)-to-serosal (blood side) flux is much greater than that predicted from the Na^+ electrochemical gradient. The transepithelial voltage was explained in terms of mucosal Na^+ and serosal K^+ diffusion potentials arranged in series, with Na^+ leaving the cells via the basolateral Na^+-K^+-ATPase (49–51, 106). This model accounted for Na^+ uptake from Na^+-rich Ringer solution but could not account for Na^+ uptake by frogs in pond water, where Na^+ would be expected to leak outward across the broad surface area of the skin epithelium. So how is Na^+ absorbed by frogs that live in ponds? It turns out that Na^+ absorption is coupled to H^+ secretion, in a 1:1 ratio (49, 50). The working hypothesis is that V-ATPase mediated proton transport across the apical membrane, and the pmf energizes the Na^+ uptake through the skin. The considerable inhibition of proton secretion by the specific V-ATPase inhibitor bafilomycin and the immunostaining of mitochondria-rich cells with antibodies to the kidney V-ATPase provide evidence for the involvement of V-ATPases (51, 106). The intracellular pH, which stimulates the insertion or retrieval of V-ATPase-containing cytoplasmic vesicles, depending on the frog's acid-base status, regulates proton secretion (109). Apically localized V-ATPase pumps the proton outward, hyperpolarizing the cell membrane facing the pond. Sodium is driven by this voltage into the cells against the unfavorable Na^+ chemical gradient. The Na^+ enters by way of amiloride-sensitive Na^+ channels located on the apical membranes of both granular and mitochondria-rich cells. Entry of Cl^- is also driven by the negative apical voltage via an electrophoretic Cl^-/HCO_3^- exchanger (95). Thus the apical V-

ATPase energizes Na^+ and Cl^- influx from the pond into amphibian skins. The basolateral Na^+-K^+-ATPase energizes the Na^+ efflux into the blood.

C. Energizing Transport Systems in Mammalian Kidney Cells by V-ATPases

It has long been known that, in addition to cleaning the body of waste products, the kidney also plays a vital role in the acid-base balance of vertebrates (13, 16, 70). An important role of the kidney is to reclaim filtered bicarbonate and to regenerate bicarbonate that is consumed during metabolic proton production (70). Proton secretion energizes both these processes, and a plasma membrane V-ATPase makes a major contribution to this essential homeostatic mechanism in several nephron segments (13, 14, 16, 31). The plasma membrane V-ATPase is situated in the proximal tubules, thick ascending limbs of Henle, distal convoluted tubules, and intercalated cells of the collecting duct system (30, 77, 171, 183). Changes in the blood pH result in V-ATPase recruitment or activation. Vesicular transport can recruit V-ATPases to specific membrane domains, and this process is physiologically regulated (15, 29, 30, 177, 202, 213). Proton-secreting α-intercalated cells express V-ATPase at their apical membrane site, and this apical distribution is increased by vesicle exocytosis during acidosis (Fig. 4). Another subtype of intercalated cell, namely the β-cell, is able to insert V-ATPases into its basolateral membrane, reversing cell polarity and increasing bicarbonate secretion. Activation appears to be mediated by cytosolic proteins that are activators or inhibitors (230, 231). It has been postulated that a 14- to 20-kDa inhibitor and a 35-kDa activator, functioning at different intracellular pH values, can contribute to V-ATPase regulation (230, 231). Finally, stimulation of proton secretion by aldosterone may be involved in both the acute and chronic regulation of V-ATPases (84, 183).

The subunits forming the functional V-ATPase molecule may vary from tissue to tissue. Isoforms may contribute to the differential targeting and transport properties as well as physiological regulation of the enzyme in different cell types and in different membrane domains. In addition, a member of the P-ATPase family (H^+-K^+-ATPase) may have a significant contribution to luminal H^+ secretion and K^+ reabsorption in the later parts of the collecting duct system and is especially active during K^+ depletion (224). Figure 4 depicts a general schematic proposal for the involvement of principal players in pH homeostasis and acid secretion by kidney epithelial cells (see Refs. 13, 14, 16, 31, 70, 83). Figure 4 stresses the pivotal function of V-ATPase, neglecting the controversial contribution of the P-type H^+-K^+-ATPase.

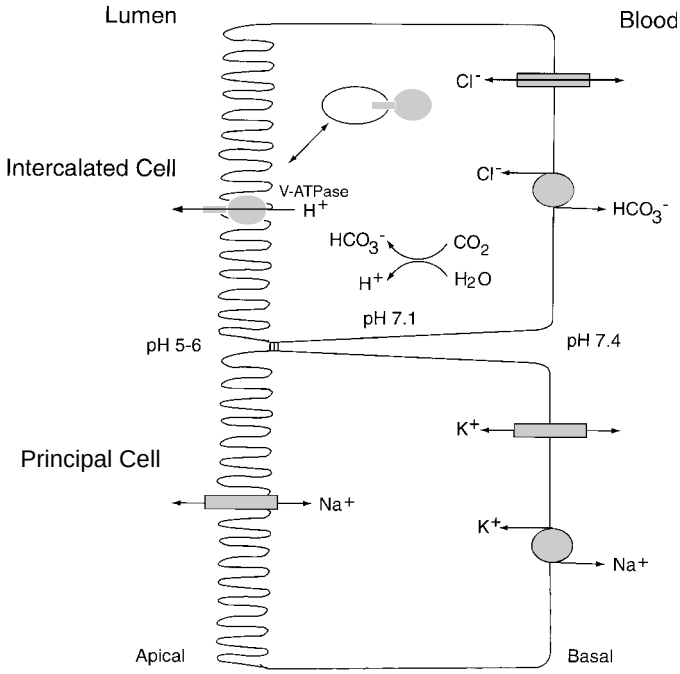

FIG. 4. Involvement of V-ATPase in acid secretion by epithelial cells in kidney, epididymis, and bladder. V-ATPase, present on apical membrane of intercalated cells, pumps protons to lumen. This generates membrane potential, negative at cytoplasmic face of membrane. In cytoplasm, protons are produced from CO_2 and water by the enzyme carbonic anhydrase, resulting in HCO_3^- secretion through the basal membrane in exchange for Cl^-. Cl^-, K^+, and Na^+ channels (depicted by a double-side arrow) function in ionic and charge balance of system.

D. Role of V-ATPase in Male Fertility

Fertilization of an egg by a single sperm consists of many steps that occur in a defined order. The precision of each step is vital to the preservation of the species, and therefore, mutations in several of the components involved in the process may cause male sterility. Vacuolar H^+-ATPases play a vital role in two key elements of the fertilization process (152). The sperm cannot fertilize an egg without proper acidification of the acrosome (128). The acrosome reaction is an exocytic event occurring when the sperm binds to the egg. The binding causes a secretion of the acrosome content as well as exposure of the inner acrosomal membrane to the egg zona pellucida. The acrosomal V-ATPase acidifies the zona pellucida at the contact point, providing a suitable pH for the activity of the secreted hydrolytic enzymes. Therefore, inhibition of V-ATPase at this stage may prevent fertilization.

The second vital function in which V-ATPase is involved is the male reproductive tract. The majority of the male reproductive tract area is maintained at an acidic pH level that ranges from 6.2 to 6.8 (113). This acidic pH may play an important role in sperm maturation and in the maintenance of immotile sperm until it is neutralized by alkaline secretions during ejaculation (8). Some cases of

male infertility may be due to the failure of the acidification mechanism. This intraluminal acidification in the epididymis and the vas deferens is mediated by specialized proton-secreting epithelial cells (28, 32). These cells have a high content of carbonic anhydrase type II, an enzyme that is also present in large amounts in renal intercalated cells (Fig. 4). Large amounts of the V-ATPase are expressed by a subpopulation of epithelial cells in the epididymis and vas deferens on their apical plasma membrane site, as well as in intracellular vesicles. By using a proton-selective extracellular microelectrode for measuring proton secretion in the vas deferens in vitro (28, 32), the contribution of these cells to reproductive tract acidification was directly demonstrated. The V-ATPase inhibitor bafilomycin inhibited the proton flux up to 80%. Thus the evolution of the epididymis includes the utilization of a V-ATPase as a major proton-secretory mechanism. Whether other transepithelial processes in the epididymis are energized by this proton gradient is not yet known.

E. V-ATPase in Mammalian Phagocytes

Phagosomes are derived from the plasma membrane, and their interior is topologically equivalent to the extracellular space. The acidification of phagosomes has been known for nearly a century (133) and is acknowledged to be essential in the response to bacterial infection. Vacuolar H^+-ATPases have been detected in the phagosomal membrane by immunohistochemistry; moreover, the acidification is ATP dependent and is virtually eliminated by the addition of bafilomycin (120, 170). The acidity of phagosomes is favorable for the catalytic function of a variety of proteases and lipases that are delivered to the phagosomal lumen by the secretory pathway. Certain bacteria, protozoans, or even viruses survive the low-pH challenge by inhibiting proton pumping by the host and/or physiological adaptations (18, 185). Consequently, the invading organisms can become long-term parasites within the frustrated phagocytic hosts, resulting in chronic and recurrent infections. In addition, V-ATPases in the membrane of phagocytes may contribute to intracellular pH homeostasis, judged by their ability to catalyze recovery from an imposed acid load (144, 193, 194). Vacuolar H^+-ATPase could also serve other functions such as providing extracellular H^+ that promotes the rapid dismutation of superoxide to hydrogen peroxide. Vacuolar H^+-ATPase may also drive metal-ion transporters that deprive the parasites of metalloproteins necessary to counteract the "oxidative challenge" launched by the invaded cell (188, 189).

F. V-ATPases Drive Bone Resorption in Mammalian Osteoclasts

Osteoclasts are specialized tissue macrophages that reside in bone; these cells are polarized, having apical and basolateral regions (12). They reabsorb mineralized bone by attaching to bone matrix and forming a tight ringlike zone of adhesion, the "sealing zone." The "bone-resorbing compartment zone" designates the extracellular space that is bordered by this ring of attachment and sandwiched between the osteoclast "apical" plasma membrane and the bone matrix. The osteoclast apical membrane is highly infolded and studded with V-ATPase (37). The bone-resorbing compartment is acidified by pumping protons across the apical membrane followed by Cl^- counterions (20). The extracellular digestion of the organic phase and the dissolution of the mineral phase of the bone matrix are mediated by lysosomal enzymes that have been secreted into the closed space. In the case of normal bone, matrix resorption by osteoclasts is tightly coupled to osteoblast matrix formation. Thus it can be said that bone resorption is one of the components of the skeleton's normal growth, remodeling, and repair processes.

Because the osteoclast is derived from a macrophage line, its plasma membrane proton pump is a V-ATPase. Antibodies to several V-ATPase subunits react specifically with the apical ruffled-border membrane (20, 211). However, the osteoclast V-ATPase may be a specialized enzyme. Proton transport activity of highly purified chicken osteoclast vesicles is more sensitive to vanadate than that mediated by other V-ATPases (37). It is 2,000 times more sensitive to the bisphosphonate tiludronate {[[(4-chlophenyl)thio]-methylene]bisphosphonate} than the kidney enzyme (42). A novel isoform of the A subunit, which is not restricted to the osteoclast and is generated by an alternative splicing event, has been cloned in chicken and may possibly be related to the variations in catalytic properties among V-ATPases (85). However, the isolation of a typical V-ATPase from ruffled membranes of chicken osteoclasts may argue against the existence of a specialized enzyme in osteoclasts (127).

In view of the fact that increased bone resorption occurs in several systemic and local diseases of the skeleton and joints, and the critical need for acidification for this resorption, therapeutic intervention now targets specific inhibition of the osteoclast proton pump. Modulation of V-ATPase activity may be suitable therapy in such widespread diseases as osteoporosis, Paget's disease, osteoarthritis, and various manifestations of skeletal cancer, as well as bone loss in periodontal disease (42). A strategy for designing drugs for osteoporosis could be modeled after the development of omeprazol as a drug against acid formation in the stomach (179). A prodrug can be obtained by a modification of V-ATPase inhibitors

such as bafilomycin A_1 or concanamycin in a way that would make them inactive for inhibition of V-ATPases. The modification would be designed to target them into the bone matrix. Vacuolar H^+-ATPase activity in osteoclasts would activate the drug to block specifically the osteoclast V-ATPase activity.

VII. FUNCTION OF VACUOLAR PROTON-ADENOSINETRIPHOSPHATASES IN pH REGULATION AND TRANSPORT PROCESSES

A. Synaptic Vesicles and Granules

Vacuolar H^+-ATPase was first detected in organelles connected with the vacuolar system. It is the main if not the only primary energy source for numerous transport systems in these organelles (155). Its function in neurotransmitter uptake and storage as well as endosomal acidification has been discussed in several reviews (82, 155). Chromaffin granules have long been one of the main objects for the study of V-ATPase. They contain up to 0.5 M monoamines such as epinephrine and norepinephrine, ~120 mM ATP, 20 mM Ca^{2+}, 5 mM Mg^{2+}, 20 mM ascorbate, as well as several proteins and enzymes. The uptake of the monoamines, ATP, and the various ions into the chromaffin granules is driven by the pmf generated by their V-ATPase (103, 151, 152). Similar vesicles storing monoamines are present in various parts of the brain. Whereas epinephrine secreted from the chromaffin granules acts on targets distant from the gland, monoamines secreted into synaptic clefts interact as neurotransmitters with specialized receptors on the adjacent postsynaptic cells. The structure and properties of the brain synaptic granule are similar to those of the chromaffin granules. Therefore, chromaffin granules are an excellent model for adrenergic granules in the brain.

Synaptic vesicles accumulate and store neurotransmitters in presynaptic cells and release them following the arrival of an action potential. The driving force for the accumulation of neurotransmitters is pmf generated by V-ATPase (103, 152). The neurotransmitter cycle is energized by two distinct ATPases. Whereas V-ATPase functions on the synaptic vesicles membrane, a Na^+-K^+-ATPase functions in energizing the plasma membrane (103). Consequently, the specific transporters for accumulation of neurotransmitters into the synaptic vesicles are driven by an electrochemical gradient of protons, and the specific transporters that function in the reuptake of neurotransmitters from the synaptic cleft are driven by an electrochemical gradient of Na^+ (103). The neurotransmission cycle is an excellent example of Mitchelian coupling between the electrochemical gradient formed by a primary ATPase and its utilization to drive secondary transport systems.

B. Lysosomes and Plant or Fungal Vacuoles

Most catalytic activities inside lysosomes require a pH of ~5, which is two pH units below that of the cytoplasm (159). The acidification of lysosomes is catalyzed by an ATP-dependent proton pump that was shown to be V-ATPase (140, 173, 176). In addition, the V-ATPase provides most of the energy required for secondary transport systems in the lysosomal membrane, some of which are vital for mammals and other higher eukaryotes. The vacuoles of plants and fungi play a major role in their osmotic regulation, storage of metabolites, Ca^{2+} homeostasis, and many other metabolic processes (107, 197). In most plant and fungal cells, the vacuole occupies over 50% of the cell volume, and their membrane contains numerous carriers, transporters, channels, and enzymes. Here, too, the V-ATPase is the major energy provider, and most secondary transport processes are driven by pmf generated by the enzyme. In plant vacuoles, there exists a backup system of proton pyrophosphatase that also generates pmf by hydrolyzing pyrophosphate (175). A pivotal function of plant and fungal vacuoles is their function in Ca^{2+} homeostasis. In contrast to mammalian cells that contain Ca^{2+}-ATPases and Ca^{2+}/Na^+ exchangers in their plasma membrane, most Ca^{2+} transport into and out of the cytoplasm of plant and fungal cells is mediated by the vacuolar membrane (3). Consequently, disruptant mutants in which the yeast V-ATPase is inactivated are sensitive to high Ca^{2+} concentrations in the medium (158, 160). Although presumably the pmf in the vacuolar system is maintained in these mutants by fluid-phase endocytosis (150), it is not sufficient to counteract Ca^{2+} flow into the cytoplasm at high external Ca^{2+} concentrations. These yeast mutants are also sensitive to low Ca^{2+} and other metal ions obtained by including 25 mM EGTA in the medium (158). This sensitivity may result from higher than normal pH inside the vacuolar system that is not sufficient for dissociating the Ca^{2+}-EGTA inside the vacuole.

C. Endosomes and Receptor Recycling

The vacuolar system consists of very complicated but highly organized machinery. Despite extensive flow of membranes among the intracellular organelles and membranes, the composition of each compartment is strictly preserved. Endo- and exocytosis are among the hallmarks of eukaryotic cells and are necessary processes for their life (129, 131). The exocytic pathway starts in the ER, but in the Golgi, it is divided into two processes of constitutive and regulated secretions (104). The constitutive secretory pathway is used for secretion of several proteins and the maintenance of the cell membrane. Synaptic vesicles and chromaffin granules are some of the organelles

of the regulated pathway; the function of the V-ATPase in these organelles has been discussed before (60). The existence of exocytosis, which adds large amounts of membrane material to the cell membrane, required the development of endocytosis that is utilized for a variety of processes. Among the best known of these processes is receptor-mediated endocytosis (33, 64). A receptor present at the cell surface binds its specific ligand in a pH-dependent step. At neutral pH, it exhibits high affinity to the ligand, but at low pH, its affinity is markedly lower. After binding of its ligand, the receptor is internalized by clathrin-coated pits into clathrin-coated vesicles. After the clathrin coat is removed, the uncoated vesicles fuse with endosomes containing V-ATPases. At the internal low pH of the endosome, generated by V-ATPase, the ligand is dissociated from its receptor, and the receptor can be either recycled to the plasma membrane or delivered into the lysosomes for distraction (64, 130, 131). This process of receptor-mediated endocytosis and pH-dependent release of the ligand is involved in numerous processes ranging from iron intake into cells through cholesterol uptake via low-density lipoproteins to the action of several receptors on the surface of eukaryotes. Inhibition of V-ATPase interferes with all of those processes.

D. Golgi Apparatus

The Golgi apparatus functions in sorting proteins of the vacuolar system and transports secreted proteins into specific delivery vesicles. Specific receptors function in the targeting of luminal and secreted proteins into their respective vesicles. Some but not all vesicles contain V-ATPases, and the *trans*-Golgi itself is furnished by the enzyme (36, 139, 229). The possible involvement of V-ATPase in the sorting of some proteins was recognized by studying the effect of amines on the targeting of vacuolar proteins (11, 174). It was shown that agents able to neutralize acidic pH in yeast vacuoles bring about the mistargetting of vacuolar enzymes. Mistargetting is also the result of treating yeast cells with specific V-ATPase inhibitors as well as mutant yeast cells in which genes encoding V-ATPase subunits are interrupted (108). In addition, the processing of the precursors of those vacuolar proteins is inhibited in the V-ATPase disruptant mutants. These mutants accumulate the intracellular precursors within the secretory pathway at some point before delivery to the vacuole and after transit to the Golgi complex (228). These precursors are accumulated at the *trans*-Golgi complex or in post-Golgi vesicles. The delivery of lysosomal enzymes from the Golgi is also dependent on the activity of V-ATPase (64, 130). Thus a picture emerges in which V-ATPase plays a key role in several activities involving the Golgi apparatus, including receptor-mediated targeting of luminal proteins. Studies involving antisense mRNA to subunit A of plant V-ATPases suggest the presence of two isozymes, one of which is localized to the Golgi apparatus (71). This observation poses an important question about the sites and mechanism of biogenesis and assembly of V-ATPases in eukaryotic cells.

E. Additional Organelles and Processes That Require V-ATPase

Vacuolar H^+-ATPase is a highly conserved enzyme, and it is assumed that all family members have a similar if not identical mechanism of action (153). A major dilemma arises: how can such a conservative enzyme function in such a wide variety of membranes and physiological processes? The principal membranes and organelles that utilize the enzyme were discussed above. Here are a few more striking examples of the diversity of V-ATPase functions. It is present in the corneal ciliary epithelium and may have a major role in regulating the eye pressure (206, 217). It may energize transport in the placenta brush border and may be involved in proton secretion into the periductular fluid in the intraphepatic bile duct epithelia (181). Vacuolar H^+-ATPase is present in the tracheal smooth muscle having the possibility to facilitate transport by P-type ATPases (164). The enzyme may have a major function in proton secretion by the turtle bladder, and it may function in Na^+ uptake by trout gills (69, 223). Vacuolar H^+-ATPase may energize water secretion by contractile vacuoles in *Dictyostelium* (59, 201). These few examples illustrated the versatility of the enzyme and open up research avenues that were not known before.

A function of V-ATPase in stress conditions is now emerging. Particularly striking are those V-ATPases that pump protons into vacuoles with an extremely low pH, approaching zero. This condition presents a mechanistic challenge in which the ATP/H^+ stoichiometry may drastically change and/or the proton slip may be canceled (137). Numerous organelles of the vacuolar system of eukaryotic cells are energized by V-ATPases, and each organelle has a specific requirement for its internal pH and membrane potential (154). The internal pH of organelles of the vacuolar system is also variable and tightly regulated. In contrast to yeast vacuoles that maintain an internal pH of ~5.5, it is believed that the vacuoles of lemon fruit may have a pH as low as 2 (141, 142, 197). Similarly, some brown and red alga maintain very low pH in their vacuolar system. Further evidence that the V-ATPase is regulated in plants is provided by the variation of vacuolar pH in different tissues of the same plant and in the same cell during the course of development or in response to changing environmental conditions. Crassulacean acid metabolism plants are the classic example of vacuolar pH regulation. The pH values of their leaf vacuoles fluctuate from pH 3 at night to pH 6 in the day.

Moreover, salt stress affects the expression of V-ATPase genes in roots and leaves (117). During the maturation of citrus lemon fruit, the vacuolar pH of the juice sac cells declines from 5.5 to ~2. To study the mechanism of hyperacidification, the kinetics of ATP-driven proton pumping by tonoplast vesicles from lemon fruits and epicotyls were compared (141, 142). Proton pumping by the fruit tonoplast-enriched membranes was chloride independent, largely insensitive to the V-ATPase inhibitors, and resistant to oxidation. The results suggested the presence of two kinds of V-ATPases (or H^+-ATPases) in the fruit. Changes in the subunit composition in V-ATPases from lemon fruits and epicotyl were detected (142).

The *Ascidian sydneiensis samea* blood contains giant cells that accumulate rare metals such as vanadium, tantalum, and niobium to extremely high concentrations (134). Recently, the biochemical properties of these cells were studied, and it was found that V-ATPase comprises ~10% of their total protein (210). The pH inside the vacuole was estimated to be ~0.1, representing a proton concentration of ~0.8 N (Y. Moriyama, personal communication). The more than 10^7 times concentration gradient of H^+ from the vacuole to the cytoplasm presents major biological questions including a mechanistic enigma for V-ATPase. Now that the subunit structure of V-ATPases is largely understood, studies on its mechanism of action and the physiology of the systems in which the enzyme is the main energy provider are likely to take center stage.

VIII. EPILOGUE

Completion of the yeast genome sequencing marks a turning point in several research areas. The sequences of all the fundamental genes encoding basic processes in the life of eukaryotic cells have been deposited in the GeneBank. Now we have to recognize them and to solve the puzzle of how they drive the biochemical and physiological processes that constitute life. This and other genome projects also mark the end of the an era of "the gene race," and put us back where we belong, as process-solving scientists. The borders among the various disciplines are collapsing, and a scientific renaissance is emerging. Although the research potential in the yeast system is far from being exhausted, the emphasis is shifting back to regulatory processes in higher organisms. The 15 genes involved in the functional assembly of the yeast V-ATPase provided an excellent framework for further studies in plants and mammals. The time is ripe for looking at factors that alter the V-ATPase activity and provide specific properties that are required for its proper function in the various organelles and membranes. The structure of V-ATPase has to be solved through high-resolution X-ray crystallography. Only then will we be able to understand the mechanism of proton pumping and the function of the various membrane subunits. Meanwhile, it is the studies of V-ATPases that function under extreme conditions; the V-ATPases that regulate complex systems, such as the secretory pathway, and V-ATPases that energize plasma membranes will present major challenges and provide high excitement. We are fortunate to be involved during these exciting times in membrane research and should be thankful to our teachers such as Efraim Racker and Peter Mitchell whose logical thinking guided us to this point.

We thank Drs. H. Wieczorek, T. Stevens, and M. Forgac for access to their reviews before publication.

The writing of the review was supported in part by a grant from the United States-Israel Binational Agricultural Research and Development Fund (to N. Nelson) and National Institute of Allergy and Infectious Diseases Research Grant AI-22444 (to W. Harvey).

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