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MINI-REVIEW

Vacuolar Proton Pumps

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Abstract

Recently a new class of proton-translocating ATPases has been localized to endomembrane compartments in plant, fungal, and mammalian cells. These proton pumps are large hetero-oligomers which have an ATP hydrolytic sector that is functionally and structurally distinct from a transmembranous proton pore. Enzymatic characteristics of these proton pumps are discussed as well as the current state of knowledge regarding subunit composition and function. In addition, recent primary sequence data are discussed which indicate that these proton pumps share a common ancestor with F_1F_0-type proton pumps of mitochondria

Key Words: Proton-translocating ATPase; organelle acidification; evolution.

Introduction

The generation and maintenance of proton gradients across biomembranes represents one of the most conserved and physiologically most universal mechanisms by which prokaryotic and eukaryotic cells energize cellular functions. Much attention has beeen focused upon F_1F_0 type ATPases, which utilize such proton gradients to energize ATP synthesis. In this review, a new class of proton pumps, which use ATP to form pH gradients, is described. These vacuolar pumps have an extraordinarily wide distribution in plant,

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fungal, and mammalian endomembranes. Particular emphasis is placed upon the clathin-coated vesicle proton pump. Although these proton pumps share certain features with F_1F_0 type ATPases, important functional and structural characteristics warrant their classification as a separate class of proton translocating systems. Characteristics of other classes of proton pumps are reviewed in order to accentuate these similarities and differences. The current status of the biochemical and structural properties of vacuolar proton pumps is discussed, as are the evolutionary relationships among F_1F_0 type ATPases and those of endomembrane compartments.

Classification of Proton Pumps

Proton-transporting mechanisms responsible for the development or utilization of proton gradients are of three types: light-driven translocators (bacteriorhodopsin), redox-energized pumps (cytochrome oxidase and the enzymes of the respiratory chain of mitochondria), and proton-translocating ATPases (Pederson and Carafoli, 1987). The latter type are now subdivided into three main groups and are compared in Table I.

E_1E_2 Type Proton Pumps

E_1E_2 type proton translocating ATPases are so named because they belong to a general class of ion-translocating ATPases which have a discernible phosphoaspartyl intermediate as part of their catalytic cycle. All E_1E_2 type ATPases are inhibited by vanadate, which substitutes for phosphate during the reaction cycle (Josephson and Cantley, 1977; O'Neal *et al.*, 1979). These pumps are composed of one catalytic subunit, in the range of 100 kDa, and in some instances (Na^+K^+ ATPases and H^+K^+ ATPase) have a second subunit of unknown function. Specific examples of E_1E_2 type proton pumps are the plasma membrane H^+ ATPase of yeast and *Neurospora* (Dame and Scarborough, 1980), the H^+K^+ ATPase present in gastric musosa (Faller *et al.*, 1982), and possibly the renal collecting duct system (Wingo, 1989).

F_1F_2 Type Proton Pumps

A second class of proton-translocating ATPases are those of the F_1F_0 variety. As shown in Table I, they are large, hetero-oligomers. Physiologically, they function as ATP synthetases and constitute the terminal step (in mitochondria and chloroplasts) of oxidative and photo phosphorylation. This directionality is in part a function of the parallel electron transport chain, which generates a proton gradient that is utilized by the F_1F_0 ATP synthetase to energize ATP synthesis. To date, there has been no discernible phospho-

Table I. Classes of Proton-Translocating ATPases

Type:	E_1E_2	F_1F_0	Vacuolar
Examples:	Plasma membrane pumps of *Neurospora* and gastric mucosa	ATP synthetase of mitochondria, chloroplasts, and bacterial membranes	Endomembranes of plant, fungal, yeast, and mammalian cells
Molecular masses of holoenzymes: (kDa):	100	350	600
Subunits:	1–2	10–12	3–9
Phosphorylated intermediate:	Yes	No	No
Inhibitors:	Vanadate	Oligomycin, efrapeptin, azide, mitochondrial ATPase, inhibitor, and DCCD	*N*-ethylmaleimide and DCCD

Table II. Subunit Composition of the Mitochondrial ATP Synthetase

Domain	Subunit	Functions
F_1 (catalytic center)	α	Required for ATP hydrolysis
	β	Contains exchangeable nucleotide sites
	γ	Required for ATP hydrolysis
	δ	Links F_1 to F_0
	ε	Links F_1 to F_0
	ATPase inhibitor	Inhibits ATPase activity
Stalk	OSCP	Links F_1 to F_0
	F_6	Links F_1 to F_0
F_0 (proton pore)	*a*	Participates in proton conduction
	b	Organizes proton pore
	c	DCCD binding proteolipid

enzyme intermediate in F_1F_0 type proton ATPases, and the energy-transducing mechanism through which proton movement is coupled to ATP synthesis (or hydrolysis) is not inhibited by vanadate (Racker, 1977).

Generally, F_1F_0 ATPase of mitochondria has three functional domains. The peripheral catalytic domain (F_1) is linked to a transmembrane proton pore (F_0) by means of a stalk assembly. In addition, a dissociable component (mitochondrial ATPase inhibitor) governs the mode of operation of the system and when attached to the complex, blocks ATP hydrolysis and thus renders the system operable in a dedicated ATP synthetic mode (Pullman and Monroy, 1963). Through biochemical resolution and reconstitution, the subunit structures of F_1F_0 ATPases have undergone elucidation. Variances exist among the subunit composition and nomenclature of F_1F_0 proton pumps of bacteria, chloroplast, and mitochondria. Shown in Table II is a list of the components of the mitochondrial ATP synthetase and their functions. The F_1 catalytic sector is composed of five subunits (α, β, γ, δ, and ε) at a stoichiometry of 3 : 3 : 1 : 1 : 1. The F_1 sector, which is extramembranous, is easily disociated from the holoenzyme by treatment with EDTA, and isolated F_1 contains all structural elements necessary for ATP hydrolysis (Racker, 1977; Senior, 1988). Subunit analysis of F_1 has revealed that ATP hydrolysis requires, at a minimum, three subunits: α, β, and γ. The α and β subunits each bind one molecule of ATP, and thus the enzyme has six binding sites (Kagawa and Nukiwa, 1981; Dunn and Futai, 1980; Dunn and Heppel, 1981). However, labeling experiments with ATP analogs reveal predominantly binding to the β subunit (Bullough and Allison, 1986; Cross *et al.*, 1987). This reflects the fact that there are significant differences in the binding affinities of ATP for its sites, and the more readily exchangeable sites are those of the β subunit

(Senior, 1988). Importantly, however, hydrolysis is viewed as requiring an interface between than α and β subunits and, in addition, the γ subunit is required to render the molecular ATP hydrolytic/synthetic site catalytically active. The remaining subunits of F_1 (δ and ε) are required for binding the catalytic sector to the proton pore F_0. Structurally, F_1 has been shown to be tethered to the lipid bilayer (and F_0) by a stalk assembly. Biochemical evidence indicates that oligomycin sensitivity conferring protein (OSCP) and F_6 are required for this functional coupling domain (Racker, 1977).

Finally, the F_0, or proton pore, component of mitochondrial ATPase is a multisubunit structure. Isolated F_0 can function as an intramembranous proton channel and is composed of three subunits, *a*, *b*, and *c*. Subunit *c* is the site of dicyclohexylcarbodiimide (DCCD) binding to F_0, and binding of DCCD blocks ATP hydrolysis and synthesis in the intact F_1F_0 complex. (Senior, 1988).

Vacuolar Proton Pumps

The third class of proton-translocating ATPases, and the focus of this discussion, is the vacuolar or endomembrane proton pumps. The names derive from the findings that these ATPases have been localized to an extremely wide array of intracellular organelles ranging from storage vacuoles of plants to numerous mammalian endomembranes. The distribution of the latter, which has been reviewed elsewhere (Stone and Xie, 1988; Sze, 1985; Mellman *et al.*, 1986; Rudnick, 1986) includes clathrin-coated vesicles, endosomes, lysosomes, synaptic vesicles, chromaffin granules, endoplasmic reticulum, Golgi membranes, tertiary granules of leukocytes, and hormonal (e.g., insulin) processing compartments. Within these endomembranes, vacuolar proton pumps carry out a diverse set of functions, ranging from the processing of endocytosed receptor–ligand complexes (transferrin in endosomes) to activation of acid hydrolases (lysosomes). From a more global standpoint, it has been demonstrated that the intraorganelle pH varies among the various endomembrane compartments, with lysosomes being the most acidic (pH = 4.8) and the trans golgi apparatus and endoplasmic reticulum being near neutrality (Mellman *et al.* 1986). It has been proposed that this interorganelle pH gradient may play an important role in the targeting of proteins within cells. In addition, these proton pumps have a distribution wider than the terms vacuolar or endomembrane suggest, in that these proton pumps are located on the plasma membrane of renal tubules, where they participate in urinary acidification (Stone and Xie, 1988; Brown *et al.*, 1987).

Initially, these proton pumps and their related ATPase activities were distinguished from the E_1E_2 type and F_1F_0 type ATPases on the basis of inhibitor sensitities. Notably endomembrane proton pumps are resistant to vanadate (and hence distinguishable from the E_1E_2 proton ATPases) and

to classic mitochondrial F_1F_0 inhibitors such as oligomycin, azide, efrapeptin, and mitochondrial ATPase inhibitor. In contrast, the vacuolar ATPases are inhibited by sulfhydryl alkylating agents such as *N*-ethylmaleimide (NEM) and dithionitrobenzoic acid (DTNB) to which the mitochondrial ATPase is resistant. Vacuolar proton pumps share with the mitochondrial ATPase a sensitivity to DCCD; however, about 100-fold greater ratios of DCCD/mg protein are required to inhibit the clathrin-coated vesicle proton pump than the ATPase of mitochondria. (Stone *et al.*, 1983).

Initial characterizations of these proton pumps in native vesicle preparations revealed several general characteristics. First, vacuolar proton pumps are electrogenic, and it has been found that activating the pump results in the generation of an interior positive potential (Xie *et al.*, 1983; Al-Awqati, 1986). As one apparent role of those pumps is endomembrane acidification, this electrical potential must be dissipated to allow for pH gradient formation. This is achieved in endomembrane preparations by the finding of a diisothiocyano-disulfonic acid stilbene (DIDS) (Xie *et al.*, 1983) and duramycin-sensitive (Stone *et al.*, 1984) chloride transporter which operates in parallel with the proton pump. This chloride transporter allows for movement of a co-ion (Cl) to achieve net electroneutrality and, hence, HCl secretion into the vesicles.

Second, most if not all, vacuolar proton pumps are directly activated, in an allosteric manner, by anions. In most instances, chloride directly activates the enzymes, whereas nitrate is inhibitory (Sze, 1985; Bennett *et al.*, 1984; Stone and Xie, 1988). The degree of anion activation varies among proton pumps, however. The beet root proton pump absolutely requires Cl for activation in native membranes (Bennett *et al.*, 1984), whereas the kidney medulla H^+ ATPase is activated only 2-fold by Cl (Kaunitz *et al.*, 1985). No cation dependence of proton pumping or related ATPase activity has been noted by numerous investigators.

Characteristics of Resolved Vacuolar Proton Pumps and ATPases

Purification of vacuolar proton pumps and/or purification of ATPase activities from native membranes containing proton pumps have been achieved over the past several years. Shown in Table III is a listing of the polypeptide composition of these isolated systems. As shown, all preparations of these enzymes contain multiple polypeptides, ranging in apparent molecular masses (determined by SDS polyacrylamide electrophoresis) from about 116 kDa to 15 kDa. When determined, the overall molecular masses of the entire complexes range from 300 to 600 kDa, and thus it has been proposed that vacuolar proton pumps are complex hetero-oligomers. Inspection of the

Table III. Polypeptide Composition of Isolated Vacuolar ATPases

Endomembrane source:	Beet[a]	Corn[b]	Oat[c]	*Neurospora*[b]	Yeast[d]	Chromaffin granules[e,f]	Clathrin-coated vesicles[g]	Kidney medulla[h]
						115	116	
Polypeptides (kDa):	67	72	72[i,j]	70[i,j]	80[i,j]	70	70[i,j]	70
	57[i]	62	60	62	64	57	58	58, 56, 53
								50, 45
						41	40	
							38	38
						33	33	33, 31
	16[k]		16[k]	15[k]	19.5	16[k]	17[k]	15, 14, 12

[a]Manolson *et al.* (1985). [b]Bowman *et al.* (1986). [c]Randall and Sze (1986). [d]Uchida *et al.* (1985). [e]Percy *et al.* (1985). [f]Moriyama and Nelson (1987). [g]Xie and Stone (1986). [h]Gluck and Caldwell (1987). [i]Denotes nucleotide binding. [j]Denotes NEM binding. [k]Denotes DCCD binding.

polypeptide components of Table III reveals that there is considerable diversity in the putative subunit structure of the enzymes. In general, those isolated from plant, fungal, and yeast membranes (Monolson *et al.*, 1985; Bowman *et al.*, 1986; Randall and Sze, 1986; Uchida *et al.*, 1985) are reported to have fewer subunits (three), whereas those isolated from mammalian sources (e.g., the clathrin-coated vesicle proton pump) have up to 8 to 13 major polypeptide components. Despite this diversity, there is a general conservation of three polypeptides — those of molecular mass of about 70, 58, and 17 kDa. As shown in Table III, characterization of resolved enzyme complexes has revealed that, in most instances, the 70-kDa component is labeled by NEM and by nucleotides or nucleotide analogues, and that the 17-kDa component is labeled by DCCD. Both of these are known inhibitors of vacuolar proton pumps, and the observations that an ATP binding site is on a polypeptide (70 kDa) of different mass than the DCCD reactive site (17 kDa) led to general speculation that these proton pumps are related to F_1F_0 type ATPases.

As discussed below, the subunit structure of vacuolar proton pump is highly controversial. In some instances, the polypeptides listed in Table III do not represent all the polypeptides visualized by SDS PAGE of isolated ATPase activities. This is particularly true of the enzymes harvested from plant and fungal sources, where limited starting materials precluded further purification. Thus it is possible in some cases that more polypeptides (and perhaps subunits), present in low copy number within these complexes, have not been identified. Possibly reflecting this lack of resolution are the wide differences in specific activities of isolated enzymes, although intrinsic differences in the turnover numbers of the various ATPases cannot be excluded at present.

In addition, only a few of the isolated enzymes have been reconstituted to demonstrate NEM-sensitive proton pumping (Xie and Stone, 1986; Moriyama and Nelson, 1987; Gluck and Caldwell, 1987). Thus it is possible in some instances that domains necessary for overall proton pump functions have been lost, and that there has been selected purification only of components necessary for ATPase activity. Detailed below are the biochemical characterizations of resolved complexes.

Biochemical Properties of Resolved Vacuolar Proton Pumps

As shown in Table III, resolution of several endomembrane proton pumps has been achieved. Purification of vacuolar proton pumps from mammalian sources (clathrin-coated vesicles, chromaffin granules) was achieved usually through solubilization with nonionic detergents ($C_{12}E_9$) and purification by means of hydroxylapatite chromatography, $(NH_4)_2SO_4$

fractionation, and glycerol gradient centrifugation (Xie and Stone, 1986; Moriyama and Nelson, 1987). Underscoring the fact that vacuolar proton pumps are integral membrane proteins was the observation that the clathrin-coated vesicle proton pump rapidly destabilized during purification and that addition of phosphatidylserine was essential for stabilization and activation after solubilization. This effect of phosphatidylserine was highly specific, and addition of other purified lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol) was not protective (Xie, *et al.*, 1984). Use of phosphatidylserine (and dithiothreitol) led to the purification of the clathrin-coated vesicle proton pump in a reconstitutively active form, and subsequently allowed for the purification of vacuolar proton translocation ATPases from other mammalian endomembranes (Moriyama and Nelson, 1987). The bovine renal medulla proton pump was isolated by a different strategy and although the resolved enzymes was lipid-activated, this was not phosphatidylserine specific (Gluck and Caldwell, 1987).

Further insight into enzyme–lipid interactions have followed from reconstitutions of purified endomembrane proton pumps. Initial reconstitution of the clathrin-coated vesicle proton pump was achieved by the freeze–thaw, cholate dilution method using liposomes prepared from ethanol-extracted lipid of bovine brain (Xie and Stone, 1986). Refinement in definition of the lipid requirements for reconstitution of this enzyme was achieved with reconstitutions performed with varying pure lipid compositions. It was found that optimal reconstitution of proton pumping was achieved when liposomes were composed of phosphatidlcholine, phosphatidylethanolamine, phosphatidylserine, and cholesterol at a mass ratio of 40 : 26 : 5 : 7.5 : 26. Surprisingly, omission of phosphatidylserine from the lipid mixture reduced proton pumping by only 10%, which is in sharp contrast to the absolute requirement for phosphatidylserine to activate the enzyme in a solubilized state. This evidently indicates that phosphatidlyserine induces a conformational change in the solubilized enzyme which mimics the structure obtained after membrane insertion (Xie *et al.*, 1986). At present, however, the sites of protein–lipid interaction have not been defined, nor have the chemical nature of probable boundary lipids been identified. Evidence for the latter comes from experiments with chromaffin granule ATPase which indicate that partially resolved enzyme can be reconstituted without exogenous lipids (Moriyama and Nelson, 1987). It should be noted that optimal reconstitution of the clathrin-coated vesicle proton pump was achieved by using a pure lipid mixture that quantitatively matched the lipid composition of native clathrin-coated vesicles (Pearse, 1976). This raises the possibility that regulation of endomembrane acidification may be achieved by bilayer composition, and that differences in the pH of various mammalian organelles may owe to intrinsic differences in the respective lipid compositions of those membranes.

In general, purified preparations of endomembrane proton pumps have biochemical features which parallel those of the native vesicle system. Thus resolved pumps are inhibited by both NEM and DCCD (Xie and Stone, 1986; Moriyama and Nelson, 1987; Sun *et al.*, 1987), although in one instance it has been reported that proton pumping is more sensitive to NEM than ATPase activity (Moriyama and Nelson, 1987). Likewise, most isolated proton pumps are activated by chloride and inhibited by nitrate. However, apparent differences have been reported, in that both ATPase and proton pumping activities of the clathrin-coated vesicle H^+ ATPase are activated by chloride, whereas only proton pumping catalyzed by the chromaffin granule ATPase is chloride stimulated (Moriyama and Nelson, 1987). The renal medulla preparation of enzyme is reportedly not chloride activated (Gluck and Caldwell, 1987), and this is in contradistinction to the observation that native vesicles from renal medulla have chloride-stimulated proton pumping activity (Kaunitz *et al.*, 1985). This discrepancy may owe to the high concentrations of chloride used in the former experiments in that stimulation for some vacuolar systems requires a lower chloride concentration and that high salt concentrations render the chromaffin granule proton pump cold labile.

As shown in Table III, most preparations of vacuolar ATPases have been found to have a nucleotide (or nucleotide analog) binding site on the 70-kDa component. Beyond this, nucleotide binding characteristics have been poorly characterized. However, it is apparent that ADP is a potent allosteric inhibitor of both the ATPase and proton pumping activities. The mode of inhibition is not kinetically a simple competitive effect, raising the likelihood that multiple nucleotide sites are present in vacuolar ATPases as in the case with F_1.

Structural Properties of Vacuolar Proton Pumps

As noted previously, labeling with NEM, nucleotides, and DCCD led to speculation that vacuolar proton pumps share a structural relationship with F_1F_0 type ATPases in that the nucleotide binding domain (70-kDa component) is structurally distinct from a low-molecular-weight DCCD binding protein. Functional evidence for the existence of two sectors, one catalytic and one serving as an intramembranous proton channel, was provided in 1984, with the finding that a partially resolved ATPase fraction could be added to ATPase-depleted clathrin-coated vesicles to restore proton pumping activity (Xie *et al.*, 1984). These experiments were reminiscent of early experiments conducted with mitochondrial ATPase, where it was shown that F_1 ATPase could be removed from submitochondrial particles to yield two

components (F_1 and membrane remnants) which were incapable of proton pumping alone, but could be reassembled to yield a proton-pumping competent system (Racker, 1977). Important differences exist, however, in that the ATP hydrolytic sector has been very difficult to remove from the vacuolar proton pumps. Generally, treatment with chaotropic agents has been required to release the putative ATP hydrolytic polypeptides (70- and 58-kDa components) and indeed, no ATPase activity has been shown in these released subunits (Arai *et al.*, 1988).

Electronmicrographic studies have provided further support for the existence of a large extramembranous sector in the vacuolar proton pump of kidney medulla. It is notable that no evident stalk assembly is visualized and the extramembranous sector appears to be in direct contact with the lipid bilayer (Brown *et al.*, 1987). It is possible that this lack of a stalk is the structural reason that dissociation of the hydrolytic sector has been difficult.

Finally, one attempt has been made to define the topography of the clathrin-coated vesicle proton pump. In this study, hydrophilic and hydrophobic probes were used to label clathrin-coated vesicles, and the labeled proton pump was immunoprecipitated. Nine polypeptides were preciptated and based on the differences in labelings, it was concluded that the 70- and 58-kDa components were extramembranous and that the 116-, 40-, 38-, 34-, 33-, 19- and 17-kDa polypeptides were intra and/or transmembranous. In addition, quantification of the molar ratios of immunoprecipitated polypeptides resulted in an apparent copy ratio of 116:70:58:40:38:34:33:19:17 at 1:3:3:1:1:1:1:1:6 (Arai *et al.*, 1988).

Subunit Composition, Structure, and Function

As has been noted earlier, the subunit composition of vacuolar proton pumps is controversial. Following is a discussion of the known characteristics of polypeptides which purify with proton pumping activites and their role in pump function. At present, there are nine to ten polypeptides present in the various preparations, and they are discussed in descending order of apparent molecular mass.

116-kDa Polypeptide

The 116-kDa polypeptide is not a constant feature of vacuolar proton pumps and indeed has been found only in preparations of the clathrin-coated vesicle and the chromaffin granule proton pumps (Xie and Stone, 1986; Moriyama and Nelson, 1987). However, it is a constant feature in these preparations. Two features of this polypeptide may explain why it is not observed in all preparations. First, the 116-kDa polypeptide is stained very

poorly with Coomassie Blue dye, and silver staining is required to reveal its existence in a density which is roughly stoichiometric with other components. Second, this polypeptide is extremely labile, and is not visualized by SDS-PAGE if samples are boiled prior to electrophoresis. Reflecting the lability of the component is its extreme protease sensitivity. Alternatively, the 116-kDa polypeptide may not be present in some vacuolar ATPases.

From a functional standpoint, removal of the 116-kDa polypeptide from the clathrin-coated vesicle proton pump results in marked functional changes. Treatment of the purified holoenzyme with the detergent nonylglucopyanoside at an alkaline pH, followed by glycerol gradient centrifugation, results in a complex which is depleted of the 116-kDa and 38-kDa components. This preparation, when resolved, cannot support Mg^{2+}-activated ATP-dependent proton pumping or ATP hydrolysis. Rather, the enzyme can only hydrolyze ATP, and ATP hydrolysis requires Ca^{2+} (1–3 mM) as a divalent cation. This reflects the effect of Ca^{2+} on the function of the native enzyme, in that this divalent cation uncouples proton pumping from ATP hydrolysis (Xie and Stone, 1988). Similar observations have been noted with mitochondrial ATPase, where it was shown that Ca^{2+} can support ATP hydrolysis but not $^{32}P_i$-ATP exchange (a function of proton pumping) (Pullman *et al.*, 1960). To date, the 116- and 38-kDa components have not been added back to the subcomplex to restore Mg^{2+}-activated functions, and the above observations remain a phenomenological association. However, we speculate that the 116-kDa (and possibly 38-kDa) polypeptide may play an important coupling function in the enzyme and that the transition from the Mg^{2+}-activatable mode may allow the pump to be turned on or off depending upon its quaternary state. This may play a role in the regulation of organelle acidification and/or preventing futile hydrolysis during the biogenesis of the pump. Such a mechanism is operable with myosin ATPase, in that myosin can only hydrolyze ATP in the presence of Ca^{2+} until it binds to actin, at which point it becomes Mg^{2+} activatable (Kiely and Martonosi, 1968).

Further controversy has surrounded the 116-kDa polypeptide of the clathrin-coated vesicle and chromaffin granule proton pumps because of the coexistence of a distinct 116-kDa ATPase in these membranes. This enzyme is an unrelated, vanadate-sensitive ATPase with unknown function. Inhibitor sensitivites and antibody analysis show that it is not present in purified preparations of either proton pump (Xie *et al.*, 1989).

Thus, at present, the 116-kDa polypeptide does not have subunit status. It has not been demonstrated to be present in all vacuolar pump preparations, and its removal from the clathrin-coated vesicle pump has not been causally linked to a change in pump function. It, however, is present at a stoichiometric copy number in the clathrin-coated vesicle proton pump, and indirect evidence suggests it may carry out a coupling function.

70- and 58-kDa Polypeptides

In contrast to the 116-kDa component, it is clear that the 70- and 58-kDa components of vacuolar proton pumps are genuine subunits which constitute part of the ATP hydrolytic center of the enzyme. As noted in Table III, both of these polypeptides are present in all proton pump preparations, and interspecies antibody cross reactivity has been shown. Beyond the fact that the 70-kDa binds nucleotides (and NEM), an antibody directed against the 70-kDa component has been shown to be inhibitory to ATPase activity (Mandala and Taiz, 1987). From a biochemical standpoint, the 70- and 58-kDa components of the clathrin-coated vesicle proton pump have been dissociated from the holoenzyme and purified. These components copurify after dissociation, which is probably reflective of native interaction within the complex. Importantly, the isolated 70–58 subcomplex cannot catalyze ATP hydrolysis, but rather requires the addition of two polypeptides (likely the 40-kDa and 33-kDa components) to render the reconstituted subunits catalytically active (Xie and Stone, 1988). This observation is reminiscent of the F_1F_0 type ATPases, where it is clear that the molecular catalytic sites are at the interface of the α and β subunits, yet a third component (γ) is required to render α and β catalytically competent (Kagawa and Nokiwa, 1981). Further support for this parallel comes from the finding that there are three copies of the 70- and 58-components in the clathrin-coated vesicle proton pump, just as there are three copies of both α and β in F_1 (Arai *et al.*, 1988).

Substantial structural knowledge of the 70- and 58-kDa components has come from the molecular cloning of the genes and *c*DNA encoding these subunits (Bowman *et al.*, 1988a,b; Zimniak *et al.*, 1989; Manolson *et al.*, 1988; Denda *et al.*, 1988a,b; Sudhof *et al.*, 1989). From these studies, several points emerge. First, there is intense phylogenetic conservation of the predicted primary structure of both components. In total, 49% of all residues are invariant between the ATPase of fungi, plants, archaebacteria and human kidney (Sudhof *et al.*, 1989). Second, there is a lesser degree of sequence homology between the 70- and 58-kDa components of the vacuolar proton pumps and the α and β subunits of *E. coli* F_1 (Sudhof *et al.*, 1989, Bowman *et al.*, 1988b). In pairwise comparisons of the sequences of the vacuolar subunits and those of α and β, it was found that identities ranged from 23 to 27%. Importantly, there was no greater homology between any two subunits when all six combinations were tested. Domains of homology between the vacuolar subunits and α and β exist in the probable nucleotide binding sites of the latter. Because of these observations, we have hypothesized that vacuolar proton pumps share a common evolutionary ancestor with the F_1 ATPase and that the primordial gene underwent duplication to give rise to two subunits in each of the divergent systems: the vacuolar proton pumps

and the F_1F_0 type ATPase (Sudhof *et al.*, 1989). Despite the finding that there are no significant differences in the degrees of homology between the vacuolar 70-kDa component and F_1 α or β, or between the vacuolar 58-kDa component and F_1 α or β, it is likely that functionally and biochemically, the 70-kDa vacuolar subunit is analogous to F_1 β and 58-kDa component is analogous to F_1 α. This obtains because of the parallels in nucleotide binding properties, in that the 70-kDa and β subunits are both readily labeled by nucleotide and nucleotide analogues, suggesting that these components contain the exchangeable (and possibly) catalytic sites.

40-, 38-, 34-, and 33-kDa Polypeptides

Little functional data are available regarding the 40-, 38-, 34-, and 33-kDa components of the clathrin-coated vesicle proton pump, other than the observation that the 40- and 33-kDa components are required (with the 70- and 58-kDa subunits) to reconstitute ATP hydrolysis (Xie and Stone, 1988). The 32-kDa component of the chromaffin-granule proton pump has been molecularly cloned. There is no sequence homology with any other protein, and the relationship of the polypeptide to that of the coated-vesicle proton pump is unknown (Wang *et al.*, 1988). In addition, a 31-kDa polypeptide present in a renal medulla proton pump preparation has been cloned. It has a small degree of sequence homology with E_1E_2-type ATPases and its function is also unknown (Hirsch *et al.*, 1988). At present it can only be speculated that some or all of these intermediate-range polypeptides, if indeed true subunits, may function to couple ATP hydrolysis to proton movement through the transmembranous proton pore.

17-kDa Polypeptide

The DCCD-binding protein (17-kDa polypeptide of clathrin-coated vesicle proton pump) has been functionally defined. When isolated from the purified holoenzyme and reconstituted, it has been shown to function as a DCCD-sensitive proton pore (Sun *et al.*, 1987). Extraction from the holoenzyme was accomplished with toluene. Because of its stability in an organic solvent and by analogy to F_0, it can be speculated that the DCCD-binding protein is a proteolipid, although covalent linkage of lipids to the protein has not been demonstrated. Resolution of the proton channel is incomplete, and at present it is not clear whether the 17-kDa subunit can function alone as a proton pore, or whether ancillary polypeptides are also required. The mRNA encoding the DCCD-binding protein of the chromaffin-granule proton pump has been cloned and sequenced, and its primary structure, which bears sequence homology to the DCCD-binding, protein (*c*) of F_0, supports the observation that this component is a subunit participating in transmembraneous proton movement (Mandel *et al.*, 1988).

Thus, to summarize subunit composition, it is clear that the 70-, 58-, and 17-kDa components of proton pump preparations are genuine subunits. In addition, pending further resolution, it is likely that the 40- and 33-kDa polypeptides of the clathrin-coated vesicle proton pump are also subunits which are required for activation of the molecular catalytic center of the 70–58 subcomplex. The role of the 116-kDa polypeptide in pump function is indirect, and there is no function attributable to the 38-, 34-, 32-, and 31-kDa polypeptides present in vacuolar proton pump preparations.

Summary

The structural properties of vacuolar pumps, with particular emphasis on the clathrin-coated vesicle system, have been reviewed. These pumps are large hetero-oligomers which have an ATP hydrolytic sector which is functionally and structurally dissociable from the transmembranous proton pore. Phylogenically, it appears that vacuolar pumps are related to F_1F_0 type ATPases through a distant, common ancestor.

Key structural issues are unresolved at present. The nature of subunit composition and functions awaits further elucidation. Important issues remain regarding the biogenesis of this nuclear-encoded heteroligomer, as well as a structural–functional analysis to explain the regulatory requirement for such complexity to achieve endomembrane acidification.

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