Journal of Bioenergetics and Biomembranes, Vol. 31, No. 1, 1999

Structure and Properties of the Clathrin-Coated Vesicle and Yeast Vacuolar V-ATPases

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Received December 1, 1998

The V-ATPases are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells. This review focuses on the the V-ATPases from clathrin-coated vesicles and yeast vacuoles. The V-ATPase of clathrin-coated vesicles is a precursor to that found in endosomes and synaptic vesicles, which function in receptor recycling, intracellular membrane traffic, and neurotransmitter uptake. The yeast vacuolar ATPase functions to acidify the central vacuole and to drive various coupled transport processes across the vacuolar membrane. The V-ATPases are composed of two functional domains. The V_1 domain is a 570-kDa peripheral complex composed of eight subunits of molecular weight 70–14 kDa (subunits A–H) that is responsible for ATP hydrolysis. The V_0 domain is a 260-kDa integral complex composed of five subunits of molecular weight 100–17 kDa (subunits a, d, c, c′ and c″) that is responsible for proton translocation. Using chemical modification and site-directed mutagenesis, we have begun to identify residues that play a role in ATP hydrolysis and proton transport by the V-ATPases. A central question in the V-ATPase field is the mechanism by which cells regulate vacuolar acidification. Several mechanisms are described that may play a role in controlling vacuolar acidification *in vivo*. One mechanism involves disulfide bond formation between cysteine residues located at the catalytic nucleotide binding site on the 70-kDa A subunit, leading to reversible inhibition of V-ATPase activity. Other mechanisms include reversible assembly and dissociation of V_1 and V_0 domains, changes in coupling efficiency of proton transport and ATP hydrolysis, and regulation of the activity of intracellular chloride channels required for vacuolar acidification.

KEY WORDS: V-ATPase; vacuolar acidification; proton transport; membrance traffic.

FUNCTION OF V-ATPases IN CLATHRIN-COATED VESICLES AND YEAST

Clathrin-coated vesicles serve a number of functions in eukaryotic cells. They are the sites of receptor clustering during receptor-mediated endocytosis and also function in targeting of newly synthesized lysosomal enzymes from Golgi to lysosomes (Forgac, 1989). Acidification also plays a similar role in both membrane traffic pathways. In endocytosis, exposure of internalized ligand–receptor complexes to a low pH activates ligand–receptor dissociation and receptor recycling to the plasma membrane (Forgac, 1989). Similarly, acidification of a late recycling compartment causes release of newly synthesized lysosomal enzymes from mannose 6-phosphate receptors, allowing the receptors to recycle to the trans-Golgi. In both cases, low pH serves as the trigger to activate ligand–receptor dissociation that is a prerequisite to receptor recycling. Acidification of endosomes has also been shown to be required for formation of endosomal carrier vesicles (which carry ligands from early to late endosomes) (Clague *et al.*, 1994; Stevens and Forgac, 1997) and to activate fusion of internalized envelope viruses (such as influenza virus) with the endosomal membrane (a step that is essential for infection).

Do the proton pumps that are responsible for acidification of these intracellular compartments derive

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0145-479X/99/0200-0057$16.00/0

from clathrin-coated vesicles? While endocytic-coated vesicles do not appear to be acidic organelles *in vivo* (Forgac, 1992), immunogold staining of endocytic-coated vesicles in MDBK cells has been observed using antibodies specific for the V-ATPases(Marquez-Sterling *et al.*, 1991). These results suggest that the proton pump in endocytic-coated vesicles may be maintained in an inactive state *in vivo* (see below). By contrast, Golgi-derived coated vesicles appear to be acidic and are thus likely the source of the proton pumps responsible for Man 6-P receptor recycling.

In neurons, the V-ATPases play an essential role in synaptic vesicles, where they provide the driving force for uptake of various neurotransmitters (Stevens and Forgac, 1997) following synaptic vesicle fusion. The protein composition of synaptic vesicles and clathrin-coated vesicles from which the clathrin coat has been removed are nearly identical (Rodman *et al.*, 1994), suggesting that coated vesicles are a precursuor to synaptic vesicles at the nerve terminal. The V-ATPase present in brain clathrin-coated vesicles is thus likely en route from the plasma membrane (following neurotransmitter release) to synaptic vesicles.

The V-ATPase in yeast serves a number of important functions in driving coupled transport of various ions and small molecules as well as in membrane traffic processes (Stevens and Forgac, 1997), but these functions will be discussed in detail elsewhere in this volume.

STRUCTURE AND SUBUNIT FUNCTION OF THE V-ATPases

Our current structural model of the V-ATPases is shown in Fig. 1. This model is based primarily on our structural studies of the bovine coated vesicle V-ATPase (Forgac, 1992), but also incorporates information on a number of smaller subunits initially identified in yeast (Stevens and Forgac, 1997). The molecular weights of the subunits for the V-ATPases from bovine coated vesicles and yeast vacuoles, together with the genes encoding these subunits in yeast, are shown in Table I.

Domain Structure and Function

As can be seen, the V-ATPase complex is composed of two structural domains. The V_1 domain is a 570-kDa peripheral complex composed of eight subunits (subunits A–H) in a stoichiometry of $A_3B_3C_1D_1E_1F_xG_yH_z$, where the stoichiometry of all but the F, G, and H subunits has been determined by quantitative amino acid analysis (Arai *et al.*, 1988). The V_1 domain is responsible for ATP hydrolysis, with the catalytic nucleotide binding sites located on the A subunits and additional nucleotide binding sites (termed "noncatalytic") located on the B subunits (see below). The V_0 domain is a 260-kDa integral complex composed of five subunits (subunits a, d, c, c′, and c″) present in a stoichiometry of $a_1d_1c''_1(c,c')_6$ (Arai *et al.*, 1988). The V_0 domain is responsible for proton translocation across the membrane (Zhang *et al.*, 1994).

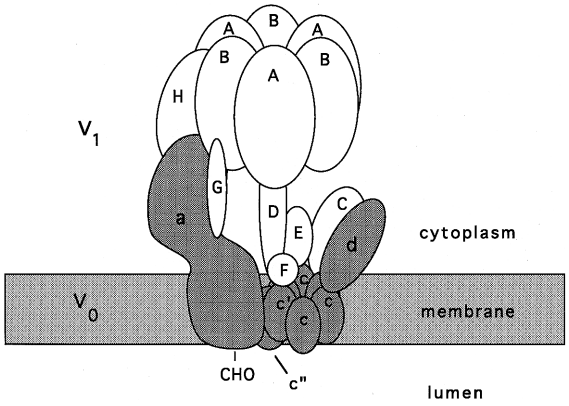

Fig. 1. Structural model of the V-ATPase. The V_1 domain (shown in white) is 570 kDa peripheral complex responsible for ATP hydrolysis while the V_0 domain (shaded) is a 260-kDa integral complex responsible for proton translocation. The existence of a second or peripheral stalk has been identified in electron micrographs of the V-ATPase from clathrin-coated vesicles (Wilkens and Forgac, unpublished observations).

The ability to dissociate the V_1 subunits from the membrane using chaotropic agents (such as KI and KNO_3) in the absence of detergents identified these subunits as peripheral (Adachi *et al.*, 1990b). Interestingly, dissociation of V_1 is activated by binding of ATP to a very high affinity site (K_d 200 n*M*) (Arai *et al.*, 1989), suggesting that nucleotide binding to the V-ATPase loosens the structure such that V_1 is more readily dissociated from V_0. Although subunit d does not possess any integral membrane spans (Wang *et al.*, 1988), it remains tightly bound to the V_0 domain upon dissociation of V_1 (Zhang *et al.*, 1992). The disposition of subunits with respect to the membrane has been analyzed using chemical modification of the V-ATPase in intact clathrin-coated vesicles by membrane impermeant reagents (Arai *et al.*, 1988). These results have indicated that all of the V_1 subunits, together with the

Table I. Subunit Composition of V-ATPases

Domain	Subunit	Coated vesicles M_r	Yeast vacuole M_r	Yeast gene	Subunit function
V_1	A	73	69	*VMA1*	Catalytic site, regulation (?)
	B	58	57	*VMA2*	Noncatalytic site, targeting (?)
	C	40	42	*VMA5*	Activity, assembly
	D	34	32	*VMA8*	Activity, assembly
	E	33	27	*VMA4*	Activity, assembly
	F	14	14	*VMA7*	Activity, assembly
	G	15	13	*VMA10*	Activity, assembly
	H	50	54	*VMA13*	Activity (not assembly)
V_0	a	100	95	*VPH1/STV1*	H^+ transport, assembly, targeting
	d	38	36	*VMA6*	Activity, assembly
	c	17	17	*VMA3*	H^+ translocation, DCCD site
	c′	17	17	*VMA11*	H^+ translocation, DCCD site (?)
	c″	19	23	*VMA16*	H^+ translocation

a and d subunits of V_0, are exposed on the cytoplasmic side of the membrane, whereas the a, c, c′, and c″ subunits have significant lumenal domains.

The proximity of subunits in the V-ATPase complex has been analyzed by cross-linking using the cleavable cross-linking reagent DTSSP followed by two-dimensional SDS-PAGE (Adachi *et al.*, 1990b). The results identified extensive contact beween the A and B subunits as well as between the C, D, and E subunits of V_1 and the c subunit of V_0, suggesting that the C, D, and E subunits form part of the bridge that structurally couples the V_1 and V_0 domains.

The V-ATPases are evolutionarily related to the F-ATPases of mitochondria, chloroplasts, and bacteria [that normally function in ATP synthesis (Weber and Senior, 1997; Fillingame, 1997; Cross and Duncan, 1996)], both in overall structure (Adachi *et al.*, 1992b) and in the sequence of specific subunits (Puopolo *et al.*, 1991, 1992a). Unlike the situation for the F-ATPases, however, the dissociated V_1 domain does not hydrolyze MgATP (Puopolo *et. al.*, 1992b) and the free V_0 domain does not act as a passive proton channel (Zhang *et al.*, 1992). Nevertheless, DCCD-inhibitable passive proton conductance has been observed for the reassembled V_0 domain (Zhang *et al.*, 1994), suggesting that this domain is responsible for proton transport. The factors regulating passive proton conductance through V_0 have not yet been elucidated.

One recently discovered feature of the V-ATPase structure is the presence of a second or peripheral stalk (Wilkens and Forgac, unpublished observations) that likely functions as a stator necessary for internal rotation of the V-ATPase molecule. The subunit composition of this stator is uncertain, but possible candidates include subunits G, H, and the N-terminal soluble domain of subunit a. The rotary mechanism (which is likely shared with the F-ATPases) is thought to be essential to coupling of proton transport and ATP hydrolysis (Vik and Antonio, 1994; Junge *et al.*, 1996).

Structure and Function of V_1 Subunits

Nucleotide Binding Subunits (A and B)

Considerable evidence has been obtained indicating that both the A and B subunits of V_1 participate in nucleotide binding. Thus, initial studies indicated that the A subunit was modified by N-ethylmaleimide (NEM) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-C1) in an ATP-protectable manner and this modification correlated with loss of activity (Arai *et al.*, 1987b). The A subunit is also labeled by 2-azido-[^{32}P]ATP such that modification of one A subunit per V-ATPase complex is sufficient to completely inactivate the enzyme (Zhang *et al.*, 1995). Modification of the A subunit by 2-azido-[^{32}P]ATP occurs at both rapidly and slowly exchangeable sites, although inhibition of activity is only associated with modification of the rapidly exchangeable site. These results suggested that the nucleotide binding site on the A subunit corresponds to the catalytic site of the enzyme.

Several approaches have been taken to characterize the structure of the nucleotide binding site on the A subunit. The first residue identified at this site was the cysteine residue responsible for sensitivity of the

V-ATPases to sulfhydryl reagents, such as NEM. Using selective (and reversible) modification by cystine (Feng and Forgac, 1992a), the key residue was identified as Cys-254 of the bovine coated vesicle V-ATPase A subunit, located in the glycine-rich consensus sequence GXGKTV. This residue is conserved as a cysteine in all A subunit sequences, but is a valine in the F-ATPase β subunit sequence. The glycine-rich loop, also termed the Walker A sequence, is seen in the X-ray crystal structure of F_1 to wrap around the terminal phosphates of ATP bound at the catalytic site on the β subunit (Abrahams *et al.*, 1994). Consistent with our assignment, we have shown that mutagenesis of the corresponding cysteine residue in the yeast V-ATPase A subunit (Cys-261) to valine results in a V-ATPase complex that is insensitive to NEM (Liu *et al.*, 1997). Interestingly, the C261V is also resistant to NBD-C1 (Liu *et al.*, 1997), suggesting that this cysteine residue is also responsible for the sensitivity of the V-ATPases to this reagent.

We also observed that Cys-254 was able to form a disulfide bond with a second cysteine residue in the same A subunit (Feng and Forgac, 1992b) and identified that residue as Cys-532 (Feng and Forgac, 1994). Cys-532 is located in the C-terminal domain of the protein and is near residues labeled by 2-azido-[^{32}P]ATP (Zhang *et al.*, 1995), suggesting that it is in close proximity to the adenine binding pocket. Interestingly, disulfide bond formation between Cys-254 and Cys-532 leads to reversible inactivation of the V-ATPase (Feng and Forgac, 1992b, 1994) and has led to our suggestion that disulfide bond formation at the catalytic site is involved in regulation of V-ATPase activity *in vivo* (see below). Disulfide bond formation does not appear to inhibit nucleotide binding to the catalytic site, however, as evidenced by 2-azido-[^{32}P]ATP labeling. Instead, we have suggested an alternative mechanism by which disulfide bond formation inhibits V-ATPase activity (Forgac, 1998). It was observed from the X-ray crystal structure of F_1 that the catalytic β subunit adopts two significantly different conformations (Abrahams *et al.*, 1994). One of these conformations (termed the "closed" conformation) is seen for the β subunit with ADP or AMP–PNP bound. The other conformation (termed the "open" conformation) has the nucleotide binding site unoccupied and differs from the closed conformation in that the C-terminal domain (possessing the adenine binding pocket) is shifted away from the central domain (containing the glycine rich loop sequence) by approximately 20 A. Each β subunit is believed to cycle between these conformations during catalysis. Because Cys-532 is located in the C-terminal domain of the V-ATPase A subunit while Cys-254 is located in the central domain, disulfide bond formation between these cysteine residues would lock the enzyme into the closed conformation, thus inhibiting catalytic activity.

To further characterize the catalytic nucleotide binding site on the A subunit, we have also carried out site-directed mutagenesis of the yeast V-ATPase A subunit (Liu *et al.*, 1997; MacLeod *et al.*, 1998). We have shown that mutation of E286 to Q, although without effect on assembly or stability of the V-ATPase complex, leads to complete loss of activity, consistent with the postulated role for the corresponding glutamic acid residue in ATP hydrolysis by the F-ATPase β subunit (Liu *et al.*, 1997). Similarly, mutation of the lysine in the glycine-rich loop sequence of the A subunit (K263) also leads to loss of activity, although a destabilization of the complex is also observed (Liu *et al.*, 1997). By contrast, mutation of a variety of other residues in the A subunit (including G250 in the glycine-rich loop and C284 and C539, two of the three conserved cysteine residues) leads to direct destabilization of the A subunit itself.

We have also investigated the effect of mutation of conserved aromatic residues in the C-terminal domain of the A subunit (MacLeod *et al.*, 1998). While mutation of F538 to W caused a threefold decrease in V_{max} and mutation of Y532 to S caused a greater than sevenfold increase in K_m, the largest effects on activity were observed for changes in F452. Mutation of F452 to A completely abolished V-ATPase activity. These results suggest that all three of these aromatic residues may play a role in formation of the adenine binding pocket on the A subunit. Finally, we have mutagenized two residues on the B subunit (Y352 and R381) postulated to be contributed by the B subunit to the catalytic sites (Liu *et al.*, 1996). Mutation of either residue to serine led to complete loss of activity, suggesting that these residues also play an important role in ATP binding or hydrolysis.

A number of lines of evidence indicate that the B subunit also partcipates in nucleotide binding. First, the B subunit, like the A subunit, is labeled by 2-azido-[^{32}P]ATP in an ATP-protectable manner (Zhang *et al.*, 1995). Labeling occurs at a rapidly exchangeable site, suggesting that, as with the F-ATPases (Abrahams *et al.*, 1994), the catalytic sites are located at the interface of the A and B subunits. The B subunit is also modified by [^{3}H]BzATP, with complete inhibition occurring upon modification of one B subunit per V-

ATPase complex (Vasilyeva and Forgac, 1996). Isolation of the labeled peptide and sequence determination localized the site of BzATP modification to the region I164 to N171. If BzATP modification of the B subunit causes inactivation of the V-ATPase, how do we know that the nucleotide binding site on the B subunit is not catalytic? The evidence for this is as follows. We have shown that nucleotide binding to the catalytc site (as measured by 2-azido-[^{32}P]ATP modification) is not blocked by disulfide bond formation or cystine modification of Cys-254, but is blocked by reaction of Cys-254 with NEM (Feng and Forgac, 1994). By contrast, BzATP modification of the B subunit is not blocked by NEM but is inhibited by disulfide bond formation or cystine modification (Vasilyeva and Forgac, 1996). These results demonstrate that the nucleotide binding site on the B subunit that is modified by BzATP is distinct from the catalytic nucleotide binding site. Nevertheless, covalent modification of this noncatalytic site by BzATP does lead to inactivation of the enzyme.

To further probe the function of the noncatalytic nucleotide binding site on the V-ATPases, we have carried out site-directed mutagenesis of both B subunit residues (Liu *et al.*, 1996) and residues postulated to be contributed by the A subunit to the noncatalytic sites (MacLeod *et al.*, 1998). In both cases, predictions were made on the basis of the available X-ray crystal structure of F_1 and sequence alignment of the nucleotide binding subunits. In contrast to mutations at the catalytic nucleotide binding sites, which had dramatic effects on activity (Liu *et al.*, 1996), changes in noncatalytic nucleotide binding site residues on the B subunit (Y370, H180, and N181) caused only partial inhibition (activities ranged from 40 to 80% of wild type). Similarly, mutation of A subunit residues predicted to be present at the noncatalytic sites (F479 and R483) also led to only partial loss of activity (30–70% of wild type) (MacLeod *et al.*, 1998). Interestingly, two A subunit mutations (R483E and R483Q) led to a time-dependent increase in V-ATPase activity following addition to the assay buffer (MacLeod *et al.*, 1998). This result may be explained by the loss in these mutants of endogenously bound nucleotide from the noncatalytic sites during purification, suggesting that occupancy of the noncatalytic sites may be required for optimal activity.

Other V_1 Subunits

As described above, covalent crosslinking studies have indicated that subunits C, D, and E of V_1 all make contact with the 17-kDa c subunit of the V_0 domain (Adachi *et al.*, 1990b), suggesting that these V_1 subunits form part of the connective stalk(s) linking V_1 and V_0. Although no sequence homology exists between the V and F-ATPases for any of the V_1 subunits, except subunits A and B, both subunits D and E have a very high predicted α helical content, suggesting that one of these polypeptides corresponds to the γ subunit. The γ subunit plays a particularly important role in F_1 by undergoing rotation within the $\alpha_3\beta_3$ hexamer during catalysis (Noji *et al.*, 1997).

We have demonstrated that it is possible to reassemble the dissociated V_1 domain back onto stripped coated vesicles containing the free V_0 domain and restore ATP-dependent proton transport (Puopolo and Forgac, 1990). We have used this *in vitro* reassembly system to evaluate the requirements for individual V_1 subunits (Puopolo *et al.*, 1992b). A stable V_1 subcomplex lacking subunit C can be isolated following dissociation of V_1 with KI and ATP and removal of the chaotrope by dialysis (Puopolo *et al.*, 1992b). We have found that readdition of this V_1(-C) subcomplex to stripped vesicles restores approximately 50% of control proton transport, although the resultant complex is unstable to detergent solubilization and density gradient sedimentation. Readdition of purified subunit C restores full activity and stability. Others have suggested an absolute requirement of Ca ATPase activity by V_1, on the presence of subunit C (see Stone *et al.*, this volume), and it is possible that some of the activity observed using the V_1(-C) complex is due to residual subunit C associated with the V_0 domain, although this would not explain the observed difference in stability of the complex before and after addition of purified subunit C.

We have also previously reported the presence in the purified V-ATPase complex of the 50-kDa subunit of the AP-2 adaptin complex (Myers and Forgac, 1993b) and the dependence on AP50 of activity and stability of the reassembled V-ATPase (Liu *et al.*, 1994). Further examination has revealed that AP50 constitutes only approximately 15% of the total protein at the 50 kDa position, the remaining protein (which is blocked at the N-terminus) corresponds to bovine subunit H, which in yeast is encoded by the *VMA13* gene (Stevens and Forgac, 1997). Thus, it is subunit H that is required for activity and stability of the reassembled V-ATPase, although both subunit H and AP50 are removed upon cystine treatment (Liu *et al.*, 1994), suggesting a communication between these proteins and the catalytic nucleotide binding site of the enzyme.

The ability to restore reassembly to a V-ATPase depleted of subunit H by addition of AP-2 also suggests the possibility that subunit H may directly associate with the AP-2 complex.

Structure and Function of V_0 Subunits

Subunits c, c′ and c″

Subunit c was initially identified as a highly hydrophobic polypeptide of 17 kDa that is responsible for the sensitivity of the V-ATPases to DCCD (Arai *et al.*, 1987a). Subsequent studies demonstrated that this subunit has sequence homology to subunit c of F_0, from which it appears to have been derived by gene dupication and fusion (Mandel *et al.*, 1988). Thus, the V-ATPase subunit c is 17 kDa and contains four transmembrane segments, with a buried glutamic acid residue in TM4 that is the site of DCCD reaction and is essential for proton transport. By contrast, the F-ATPase subunit c contains two transmembrane helixes with the essential buried carboxyl group in TM2 (Fillingame, 1997). Interestingly, subunit c appears to contribute the same number of transmembrane helixes (24) to the integral domain for both V- and F-ATPases, since there are 6 copies of this subunit in V_0 (Arai *et al.*, 1988) as compared wth 12 copies in F_0 (Fillingame, 1997). This means that there are one-half as many buried carboxyl groups in the V_0 domain as in F_0, leading to the suggestion that this structural difference is responsible for the difference in H^+/ATP stoichiometry of the V- and F-ATPases (Cross and Taiz, 1990). Interestingly, in both cases, reaction of a single subunit c with DCCD is sufficient to completely block proton transport by the complex (Arai *et al.*, 1987a).

Unlike the case of F_0, the V_0 domain contains two additional proteolipid subunits, both of which have homology to subunit c (Stevens and Forgac, 1997). Subunit c′ also contains four transmembrane segments with the buried carboxyl in TM4 while subunit c″ contains five transmembrane segments with the buried carboxyl in TM3. While it is unclear why the V-ATPases require three proteolipid subunits, genetic studies in yeast have demonstrated that each V-ATPase complex must contain at least one copy of each proteolipid subunit (Hirata *et al.*, 1997).

Reassembly studies of V_0 have revealed that while low levels of DCCD-inhibitable passive proton conduction are observed with the c (and c′) subunits alone, maximal proton conduction requires the presence of the remaining V_0 subunits (a,d,c″) as well (Zhang *et al.*, 1994).

Subunit a

Subunit a is a 100-kDa transmembrane glycoprotein (Adachi *et al.*, 1990b) that contains an amino terminal hydrophilic domain and a carboxyl terminal hydrophobic domain containing 6–7 putative transmembrane segments (Manolson *et al.*, 1992). Reassembly and reconstitution studies have revealed that it aids in proton conduction by the V_0 domain (Zhang *et al.*, 1994) and that it possesses a binding site for the specific V-ATPase inhibitor bafilomycin (Bowman *et al.*, 1988). In yeast, subunit a is encoded by two homologous genes (*VPH1* and *STV1*), which encode proteins that are targeted to different intracellular destinations. Thus, Vph1p is targeted to the vacuole, while Stv1p is targeted to some other intracellular compartment, possibly endosomes or Golgi (Manolson *et al.*, 1994). We have investigated the role of subunit a in proton transport and assembly of the V-ATPase complex using a combination of site-directed and random mutagenesis. Our initial studies revealed that mutation of three charged residues buried in the transmembrane sector (E789, H743, and K593) lead to significant decreases in activity that are not attributable to decreases in stability or assembly of the V-ATPase complex (Leng *et al.*, 1996). These studies suggested that the 100-kDa subunit is the functional V-ATPase homolog to the F-ATPase a subunit, which plays a crucial role in proton conductance by F_0 (Cain and Simoni, 1988). More recent studies have revealed that while charged residues at positions 789 and 743 of Vph1p are not absolutely required for proton transport, both residues are in a position to significantly influence activity, as revealed by the altered pH dependence of proton transport for all of the mutations at these two positions (Leng *et al.*, 1998). These results suggest that buried charged residues in the C-terminal transmembrane segments of subunit a play a role in proton conductance, possibly by allowing protons to reach the buried carboxyl groups of subunit c.

A number of additional mutations effecting activity were identified by random mutagenesis (Leng *et al.*, 1998). Thus, mutation of either L739 or L746 to S led to almost complete loss of activity, possibly by turning the helix containing H743 away from the proton conduction pathway. Similarly, mutation of H729 to R in the loop preceding the penultimate transmem-

brane segment caused inhibition of >90% of proton transport activity. By contrast, a cluster of five mutations in the region L800 to G814 led to loss of assembly, suggesting that this region at the C-terminus of the a subunit plays an important role in the interaction between the V_1 and V_0 domains (Leng *et al.*, 1998).

Subunit d

Subunit d is unique among the V_0 subunits in possessing no putative transmembrane helixes (Wang *et al.*, 1988), but in remaining tightly bound to the V_0 domain (Zhang *et al.*, 1992), presumably through protein–protein interactions with other V_0 subunits. Although subunit d has been shown to be exposed on the cytoplasmic side of the membrane (Adachi *et al.*, 1990a), nothing is known concerning its function.

REGULATION OF VACUOLAR ACIDIFICATION

Considerable evidence has accumulated that cells are able to maintain distinct intracellular compartments at different pH and this is clearly important in the role that vacuolar acidification plays in various cellular processes (Stevens and Forgac, 1997). Several mechanisms have been proposed for how vacuolar acidification is controlled *in vivo*; several of these are discussed below.

Disulfide Bond Formation at the Catalytic Site

As described above, we had previously shown that disulfide bond formation between Cys-254 and Cys-532 of the bovine V-ATPase A subunit leads to reversible inactivation of the V-ATPase in clathrin-coated vesicles (Feng and Forgac, 1992b, 1994). Moreover, we found that a significant fraction of the V-ATPase in native clathrin-coated vesicles exists in this reversibly inactivated, disulfide-bonded state (Feng and Forgac, 1992b). To test whether this disulfide bond formation was due to oxidation of the V-ATPase during isolation of coated vesicles, we monitored the disulfide bonded state of the enzyme for various times after isolation of the vesicles in the absence of reducing agents or molecular oxygen. We find that, even in the absence of reducing agents, the fraction of Cys-254 in the reduced form increases with time, indicating that reduction of the Cys-254–Cys-532 disulfide bond comes about via thiodisulfide exchange involving a third A subunit cysteine residue that exists as a free sulfhydryl (Feng and Forgac, 1994). Thus, the 50% of the enzyme that is observed in the oxidized state after the 1-day isolation procedure is actually a lower estimate and the fraction that exists in this state in clathrin-coated vesicles *in vivo* is likely significantly higher. This is consistent with the observation that endocytic-coated vesicles *in vivo* do not appear to be acidic organelles (Forgac, 1992), despite the presence of immunoreactive V-ATPase (Marquez-Sterling *et al.*, 1991). Interestingly, the V-ATPase in synaptic vesicles, which would be expected to be constitutively active in neuronal cells, is in the fully reduced state, even when isolated under the same conditions as the coated vesicle enzyme (Rodman *et al.*, 1994).

We have conducted a preliminary test of the role of disulfide bond formation in regulation of vacuolar pH in the Golgi using yeast (Liu *et al.*, 1997). In *vma*$^-$ mutants lacking a functional V-ATPase, a delay and partial missorting in delivery of newly synthesized vacuolar proteins from the Golgi to the vacuole is observed (Stevens and Forgac, 1997). This suggests that vacuolar acidification plays a role in vacuolar protein targeting in yeast. If this role is similar to that in targeting of newly synthesized lysosomal proteins in mammalian cells (see first section), expression of a constitutively active V-ATPase in the Golgi might cause a similar missorting phenotype in yeast. The C261V A subunit mutant that is unable to form the inhibitory disulfide bond at the active site would be predicted to give rise to such a constitutively active enzyme. We observed that while this mutant was, in fact, resistant to inhibition by NEM or oxidation, no change was observed in the targeting of the soluble vacuolar protein carboxypeptidase Y (Liu *et al.*, 1997). This suggests that disulfide bond formation may not be regulating V-ATPase activity in the Golgi, at least in yeast. Alternatively, vacuolar acidification may be playing a somewhat different role in targeting in yeast and mammalian cells. Interestingly, results on analysis of *CYS4* mutants that show altered redox potential as a result of a defect in cysteine biosynthesis suggest that disulfide bond formation can play a role in regulating V-ATPase activity *in vivo* (Oluwatosin and Kane, 1997).

Most recently, we have demonstrated that disulfide bond formation at the catalytic site of the V-ATPase can be induced by the nitric oxide-generating

reagent *S*-nitrosoglutathione (SNG) (Forgac, 1999). While the concentrations of SNG required for inhibition are relatively high ($K_{0.5}$ 200–400 θ μ), suggesting that this does not represent a means of globally changing V-ATPase activity in the cell, it is possible that the concentration of nitric oxide (or the glutathione adduct) may become high enough in the vicinity of an activated nitric oxide synthase to effect the activity of nearby V-ATPase molecules. In fact, nitric oxide synthases localized to particular sites within the cell (for example, the plasma membrane) have been observed and could be responsible for maintaining V-ATPases in the corresponding membranes in an inactive state. Interestingly, once formed, the inhibitory disulfide bond is not readily reduced by intracellular levels of reduced glutathione (Forgac, 1999), suggesting that cleavage may in fact occur through a thiodisulfide bond rearrangement, as was originally postulated (Feng and Forgac, 1994).

Reversible Association and Dissociation of V_1 and V_0

Kane has demonstrated that in yeast, glucose deprivation results in a rapid dissociation of V_1 and V_0 domains and that this effect is reversed upon readdition of glucose (Kane, 1995). This suggests that there is a dynamic equilibrium between assembled and dissociated V-ATPase complexes that plays a role in controlling vacuolar acidification in yeast. A similar conclusion has been reached from studies of changes in V-ATPase assembly during molting in insects (Sumner *et al.*, 1995).

In mammalian cells we have found evidence for the existence of free V_1 domains in the cytoplasm of MDBK cells (Myers and Forgac, 1993a). We also observe the presence of a significant population of free V_0 domains in clathrin-coated vesicle membranes (Zhang *et al.*, 1992). Interestingly, free V_1 domains are not active in hydrolysis of MgATP (Puopolo *et al.*, 1992b) and free V_0 domains are not normally functional as passive proton channels (Zhang *et al.*, 1992), as expected if significant populations of these free complexes exist in the cell. Whether a dynamic equilibrium between assembled and dissociated V-ATPase complexes exists in mammalian cells similar to that observed in yeast and insect cells remains to be determined.

Changes in Coupling Efficiency of Proton Transport and ATP Hydrolysis

We have observed that a variety of conditions cause a change in the efficiency of coupling of proton transport and ATP hydrolysis by the V-ATPases. Thus, solubilization of the V-ATPase with detergent gives rise to an enzyme whose ATPase activity is not inhibited by DCCD, despite the physical attachment of the V_1 and V_0 domains and reaction of the solubilized protein with DCCD (Arai *et al.*, 1987a). High concentrations of ATP cause a partial uncoupling of the V-ATPase in which ATP hydrolysis continues to increase, but proton transport plateaus and then decreases with increasing concentration (Arai *et al.*, 1989). Mild proteolysis also causes a partial uncoupling of the V-ATPase, since treatment with low concentrations of trypsin causes a complete loss of proton transport despite the continued presence of 50% of control levels of ATP hydrolysis (Adachi *et al.*, 1990a). Most recently we have found that sodium azide is able to completely inhibit proton transport by the V-ATPase without effecting ATP hydrolysis, despite the fact that it is not acting as a proton ionophore (Vasilyeva and Forgac, 1998). These results suggest that the V-ATPase is poised to show an altered coupling of proton transport and ATP hydrolysis and that this may represent an important mechanism of controlling vacuolar acidification *in vivo*. The normal intracellular signals that effect this coupling efficiency, however, remain to be identified.

Changes in Chloride Channel Activity

We have demonstrated that proton transport by the coated vesicle V-ATPase is an electrogenic process, requiring the movement of another charged species to dissipate the membrane potential established during proton transport (Arai *et al.*, 1989). We also showed that in coated vesicles this dissipation of the membrane potential is accomplished by a chloride channel that is distinct from the V-ATPase (Arai *et al.*, 1989). Moreover, we observed that the activity of this chloride channel is regulated by phosphorylation by cAMP-dependent protein kinase (Mulberg *et al.*, 1991). Thus, dephosphorylation of the chloride channel decreased chloride conductance and ATP-dependent acidification while phosphorylation by PKA increased both activities. These results suggest that changes in the activity of a chloride channel required for vacuolar acidifica-

tion represents an important mechanism by which the pH of intracellular compartments can be controlled.

ACKNOWLEDGMENT

This work was supported by National Institute of Health Grant GM34478.

REFERENCES

Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994). *Nature* **370**, 621–628.
Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1990a). *J. Biol. Chem.* **265**, 960–966.
Adachi, I., Puopolo, K., Marquez-Sterling, N., Arai, H., and Forgac, M. (1990b). *J. Biol. Chem.* **265**, 967–973.
Arai, H., Berne, M., and Forgac, M. (1987a). *J. Biol. Chem.* **262**, 11006–11011.
Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., and Forgac, M. (1987b). *Biochemistry* **226**, 6632–6638.
Arai, H., Terres, G., Pink, S., and Forgac, M. (1988). *J. Biol. Chem.* **263**, 8796–8802.
Arai, H., Pink, S., and Forgac, M. (1989). *Biochemistry* **28**, 3075–3082.
Bowman, E. J., Siebers, A., and Altendorf, K. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 7972–7976.
Cain, B. D., and Simoni, R. D. (1988). *J. Biol. Chem.* **263**, 6606–6612.
Clague, M. J., Urbe, S., Aniento, F., and Gruenberg, J. (1994). *J. Biol. Chem.* **269**, 21–24.
Cross, R. L., and Duncan, T. M. (1996). *J. Bioenerg. Biomembr.* **28**, 403–408.
Cross, R. L., and Taiz, L. (1990). *FEBS Lett.* **259**, 227–229.
Feng, Y., and Forgac, M. (1992a). *J. Biol. Chem.* **267**, 5817–5822.
Feng, Y., and Forgac, M. (1992b). *J. Biol. Chem.* **267**, 19769–19772.
Feng, Y., and Forgac, M. (1994). *J. Biol. Chem.* **269**, 13224–13230.
Fillingame, R. H. (1997). *J. Exp. Biol.* **200**, 217–224.
Forgac, M. (1989). *Physiol. Rev.* **69**, 765–796.
Forgac, M. (1992). *J. Bioenerg. Biomembr.* **24**, 341–350.
Forgac, M. (1998). *Advan. Mol. Cell Biol.* **23B**, 403–453.
Forgac, M. (1999). *J. Biol. Chem.* **274**, 1301–1305.
Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997). *J. Biol. Chem.* **272**, 4795–4803.
Junge, W., Sabbert, D., and Engelbrecht, S. (1996). *Ber. Bunsenges.-Phys. Chem.* **100**, 2014–2019.
Kane, P. M. (1995). *J. Biol. Chem.* **270**, 17025–17032.
Leng, X. H., Manolson, M., Liu, Q., and Forgac, M. (1996). *J. Biol. Chem.* **271**, 22487–22493.
Leng, X. H., Manolson, M., and Forgac, M. (1998). *J. Biol. Chem.* **273**, 6717–6723.
Liu, Q., Feng, Y., and Forgac, M. (1994). *J. Biol. Chem.* **269**, 31592–31597.
Liu, Q., Kane, P. M., Newman, P. R., and Forgac, M. (1996). *J. Biol. Chem.* **271**, 2018–2022.
Liu, Q., Leng, X. H., Newman, P., Vasilyeva, E., Kane, P. M., and Forgac, M. (1997). *J. Biol. Chem.* **272**, 11750–11756.
MacLeod, K.J., Vasilyeva, E., Baleja, J. D., and Forgac, M. (1998). *J. Biol. Chem.* **273**, 150–156.
Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y. C., Nelson, H., and Nelson, N. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 5521–5524.
Manolson, M. F., Proteau, D., Preston, R. A., Stenbit, A., Roberts, B. T., Hoyt, M., Preuss, D., Mulholland, J., Botstein, D., and Jones, E. W. (1992). *J. Biol. Chem.* **267**, 14294–14303.
Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994). *J. Biol. Chem.* **269**, 14064–14074.
Marquez-Sterling, N., Herman, I. M., Pesecreta, T., Arai, H., Terres, G., and Forgac, M. (1991). *Eur. J. Cell Biol.* **56**, 19–33.
Mulberg, A. E., Tulk, B. M., and Forgac, M. (1991). *J. Biol. Chem.* **266**, 20590–20593.
Myers, M., and Forgac, M. (1993a). *J. Cell. Physiol.* **156**, 35–42.
Myers, M., and Forgac, M. (1993b). *J. Biol. Chem.* **268**, 9184–9186.
Noji, H., Yasuda, R., Yoshida, M., and Kazuhiko, K (1997). *Nature* **386**, 299–302.
Oluwatosin, Y. E., and Kane, P. M. (1997). *J. Biol. Chem.* **272**, 28149–28157.
Puopolo, K., and Forgac, M. (1990). *J. Biol. Chem.* **265**, 14836–14841.
Puopolo, K., Kumamoto, C., Adachi, I., and Forgac, M. (1991). *J. Biol. Chem.* **266**, 24564–24572.
Puopolo, K., Kumamoto, C., Adachi, I., Magner, R., and Forgac, M. (1992a). *J. Biol. Chem.* **267**, 3696–3706.
Puopolo, K., Sczekan, M., Magner, R., and Forgac, M. (1992b). *J. Biol. Chem.* **267**, 5171–5176.
Rodman, J., Feng, Y., Myers, M., Zhang, J., Magner, R., and Forgac, M. (1994). *Ann. N.Y. Acad. Sci.* **733**, 203–211.
Stevens, T. H. and Forgac, M. (1997). *Annu. Rev. Cell Develop. Biol.* **13**, 779–808.
Sumner, J. P., Dow, J. A., Early, F. G., Klein, U., Jager, D., and Wieczorek, H. (1995). *J. Biol. Chem.* **270**, 5649–5653.
Vasilyeva, E., and Forgac, M. (1996). *J. Biol. Chem.* **271**, 12775–12782.
Vasilyeva, E., and Forgac, M. (1998). *J. Biol. Chem.* **273**, 23823–23829.
Vik, S. B., and Antonio, B. J. (1994). *J. Biol. Chem.* **269**, 30364–30369.
Wang, S. Y., Moriyama, Y., Mandel, M., Hulmes, J. D., Pan, Y. C., Danho, W., Nelson, H., and Nelson, N. (1988). *J. Biol. Chem.* **263**, 17638–17642.
Weber, J., and Senior, A. E. (1997). *Biochim. Biophys. Acta* **1319**, 19–58.
Zhang, J., Myers, M., and Forgac, M. (1992). *J. Biol. Chem.* **267**, 9773–9778.
Zhang, J., Feng, Y., and Forgac, M. (1994). *J. Biol. Chem.* **269**, 23518–23523.
Zhang, J., Vasilyeva, E., Feng, Y., and Forgac, M. (1995). *J. Biol. Chem.* **270**, 15494–15500.