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STRUCTURE, MECHANISM AND REGULATION OF THE CLATHRIN-COATED VESICLE AND YEAST VACUOLAR H^+-ATPases

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Summary

The vacuolar H^+-ATPases (or V-ATPases) are a family of ATP-dependent proton pumps that carry out acidification of intracellular compartments in eukaryotic cells. This review is focused on our work on the V-ATPases of clathrin-coated vesicles and yeast vacuoles. The coated-vesicle V-ATPase undergoes trafficking to endosomes and synaptic vesicles, where it functions in receptor recycling and neurotransmitter uptake, respectively. The yeast V-ATPase functions to acidify the central vacuole and is necessary both for protein degradation and for coupled transport processes across the vacuolar membrane.

The V-ATPases are multisubunit complexes composed of two functional domains. The V_1 domain is a 570 kDa peripheral complex composed of eight subunits of molecular mass 73–14 kDa (subunits A–H) that is responsible for ATP hydrolysis. The V_o domain is a 260 kDa integral complex composed of five subunits of molecular mass 100–17 kDa (subunits a, d, c, c′ and c″) that is responsible for proton translocation. To explore the function of individual subunits in the V-ATPase complex as well as to identify residues important in proton transport and ATP hydrolysis, we have employed a combination of chemical modification, site-directed mutagenesis and *in vitro* reassembly.

A central question concerns the mechanism by which vacuolar acidification is controlled in eukaryotic cells. We have proposed that disulfide bond formation between conserved cysteine residues at the catalytic site of the V-ATPase plays an important role in regulating V-ATPase activity *in vivo*. Other regulatory mechanisms that are discussed include reversible dissociation and reassembly of the V-ATPase complex, changes in the tightness of coupling between proton transport and ATP hydrolysis, differential targeting of V-ATPases within the cell and control of the Cl^- conductance that is necessary for vacuolar acidification.

Key words: V-ATPase, clathrin-coated vesicle, yeast, vacuole, structure, mechanism, regulation.

Function of V-ATPases in clathrin-coated vesicles and yeast

Clathrin-coated vesicles serve a number of functions in animal cells. They function as the site of ligand–receptor clustering during the process of receptor-mediated endocytosis and also serve in targeting of newly synthesized lysosomal enzymes from the Golgi complex to lysosomes (Mellman, 1996). These functions are aided by an associated family of proteins (the adaptins) that recognize and bind the cytoplasmic tails of receptors undergoing cellular trafficking. Vacuolar acidification also plays a similar role in both endocytosis and intracellular membrane traffic (Stevens and Forgac, 1997). During endocytosis, exposure of internalized ligand–receptor complexes to low pH activates ligand–receptor dissociation and receptor recycling to the plasma membrane. During intracellular targeting of lysosomal enzymes, exposure of complexes of lysosomal enzymes and the mannose-6-phosphate receptor to low pH activates release of the lysosomal enzymes and recycling of receptors to the *trans*-Golgi. In both cases, low pH serves to trigger the ligand–receptor dissociation that is necessary for receptor recycling. In addition, endosomal acidification is required for the formation of endosomal carrier vesicles, which are involved in the transport of ligands from early to late endosomes (Clague et al., 1994), and to activate the fusion of internalized envelope viruses (such as influenza virus) with the endosomal membrane, a step that is essential for viral infection.

An important question is whether the proton pumps that acidify these early and late recycling compartments are derived from clathrin-coated vesicles. Although endocytotic coated vesicles do not appear to be acidic organelles *in vivo* (Forgac, 1992), the presence of V-ATPases in endocytotic coated vesicles has been observed by immunoelectron microscopy (Marquez-Sterling et al., 1991). This observation suggests that the V-ATPases that are present in endocytotic coated vesicles are maintained in an inactive state (see below). By contrast, Golgi-derived coated vesicles appear to be acidic organelles *in vivo* (Anderson and Orci, 1988), and thus are likely to be the

source of the proton pumps that are involved in intracellular targeting of lysosomal enzymes.

In neurons, the V-ATPases play an essential role in synaptic vesicles, where they provide the driving force for the uptake of various neurotransmitters (Moriyama et al., 1992). Following the fusion of synaptic vesicles at the nerve terminal, clathrin-coated vesicles function in the retrieval of proteins (including presumably the V-ATPase) from the presynaptic membrane. Coated vesicles in the neuron are thus a precursor to synaptic vesicles, as supported by the similarity of their complement of membrane proteins (Rodman et al., 1994). The V-ATPase present in brain clathrin-coated vesicles is thus probably *en route* from the plasma membrane (following neurotransmitter release) to synaptic vesicles.

The V-ATPase in yeast functions to drive coupled transport of small molecules and ions across the vacuolar membrane (Anraku et al., 1992), to provide the acidic environment that is required for protein degradation in the vacuole and in an as yet undefined step in intracellular targeting of vacuolar proteins (Stevens and Forgac, 1997).

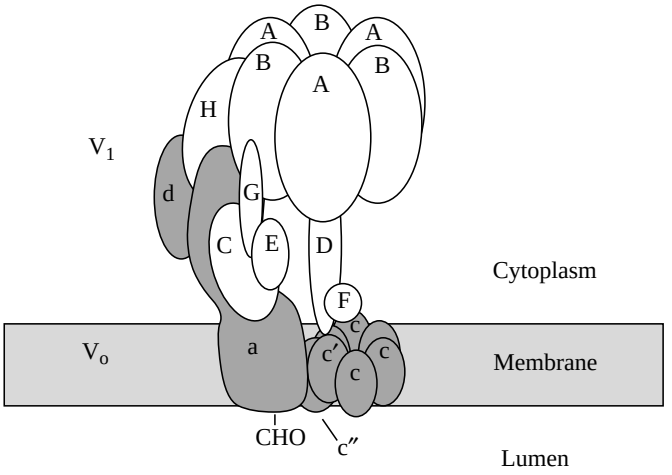

Fig. 1. Structural model of the V-ATPase. The V_1 domain (shown in white) is a 570 kDa peripheral complex responsible for ATP hydrolysis, whereas the V_o domain (shaded) is a 260 kDa integral complex responsible for proton translocation. A second or peripheral stalk has been identified in electron micrographs of the V-ATPase of *Clostridium fervidus* (Boekema et al., 1997) and clathrin-coated vesicles (S. Wilkens and M. Forgac, unpublished observations). For details of subunits, see text and Table 1.

Structure and subunit function of the V-ATPases

Our current structural model of the V-ATPases is shown in Fig. 1. This model incorporates information derived from structural studies of the bovine coated-vesicle V-ATPase (Forgac, 1992) as well as information obtained on the V-ATPases from several other sources, particularly yeast (Stevens and Forgac, 1997). Table 1 shows the molecular masses of the subunits of the bovine coated-vesicle and yeast vacuolar ATPases as well as the genes encoding the V-ATPase subunits in yeast.

Domain structure and function

As can be seen in Fig. 1, 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_o 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_o domain is

Table 1. *Subunit composition of V-ATPases*

Domain	Subunit	Coated vesicles (kDa)	Yeast vacuole (kDa)	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_o	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

DCCD, dicyclohexylcarbodiimide.

responsible for proton translocation across the membrane (Zhang et al., 1994).

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 nmol l^{-1}) (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_o. Although subunit d does not possess any integral membrane spans (Wang et al., 1988), it remains tightly bound to the V_o 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 the V_1 subunits, together with the a and d subunits of V_o, are exposed on the cytoplasmic side of the membrane, whereas the a, c, c′ and c″ subunits have significant luminal domains.

The proximity of subunits in the V-ATPase complex has been analyzed by crosslinking, using the cleavable crosslinking reagent 3,3′-dithiobis(sulfosuccinimidylpropionate) (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 what was initially thought to be the c subunit of V_o. Subsequent analysis has demonstrated that the contacts identified were actually between subunits D or E and subunits F or G, which have a very similar mobility to subunit c on SDS–PAGE (T. Xu and M. Forgac, in preparation). Co-immunoprecipitation using a monoclonal antibody to subunit E also demonstrated that subunits C and E are in contact (Puopolo et al., 1992b).

The V-ATPases are evolutionarily related to the F-ATPases of mitochondria, chloroplasts and bacteria (which normally function in ATP synthesis; Weber and Senior, 1997; Fillingame, 1997; Cross and Duncan, 1996), both in overall structure (Adachi et al., 1990b) and in the sequence of specific subunits (Puopolo et al., 1991, 1992a). Unlike the dissociated F_1 domain, however, the dissociated V_1 domain does not hydrolyze MgATP (Puopolo et al., 1992b), and the free V_o domain does not act as a passive proton channel (Zhang et al., 1992). Nevertheless, a dicyclohexylcarbodiimide (DCCD)-inhibitable passive proton conductance has been observed for the reassembled V_o domain (Zhang et al., 1994), suggesting that this domain is responsible for proton transport. The factors regulating passive proton conductance through V_o have not yet been elucidated.

A second or peripheral stalk has recently been reported in a Na^+-dependent V-ATPase from *Clostridium fervidus* (Boekema et al., 1997) and in the V-ATPase from clathrin-coated vesicles (S. Wilkens and M. Forgac, in preparation). The subunit composition of this putative stator is uncertain, but possible candidates include subunits G and H and the N-terminal soluble domain of subunit a. It has been proposed that a peripheral stalk is essential for the rotary mechanism coupling proton transport to ATP hydrolysis by F-ATPases (Cross and Duncan, 1996; Junge et al., 1996; Vik and Antonio, 1994); a similar mechanism is likely to apply to the V-ATPases as well (see below).

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 the V_1 domain 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-Cl) in an ATP-protectable manner and that 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 inactivate the enzyme completely (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 the 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 for the bovine coated-vesicle V-ATPase was identified as Cys254 of the A subunit, located in the glycine-rich consensus sequence G*X*GKTV. This residue is conserved as a cysteine in all A subunit sequences, but a valine is present at this site 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 phosphate of ATP that is 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 (Cys261) to valine results in a V-ATPase complex that is insensitive to NEM (Liu et al., 1997). The Cys261Val mutant is also resistant to NBD-Cl (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 Cys254 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 Cys532 (Feng and Forgac, 1994). Cys532 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. Disulfide bond formation between Cys254 and Cys532 leads to reversible inactivation of the V-ATPase (Feng and Forgac, 1992b, 1994), suggesting that disulfide bond formation at the catalytic site is involved in the regulation of V-ATPase activity *in vivo* (see

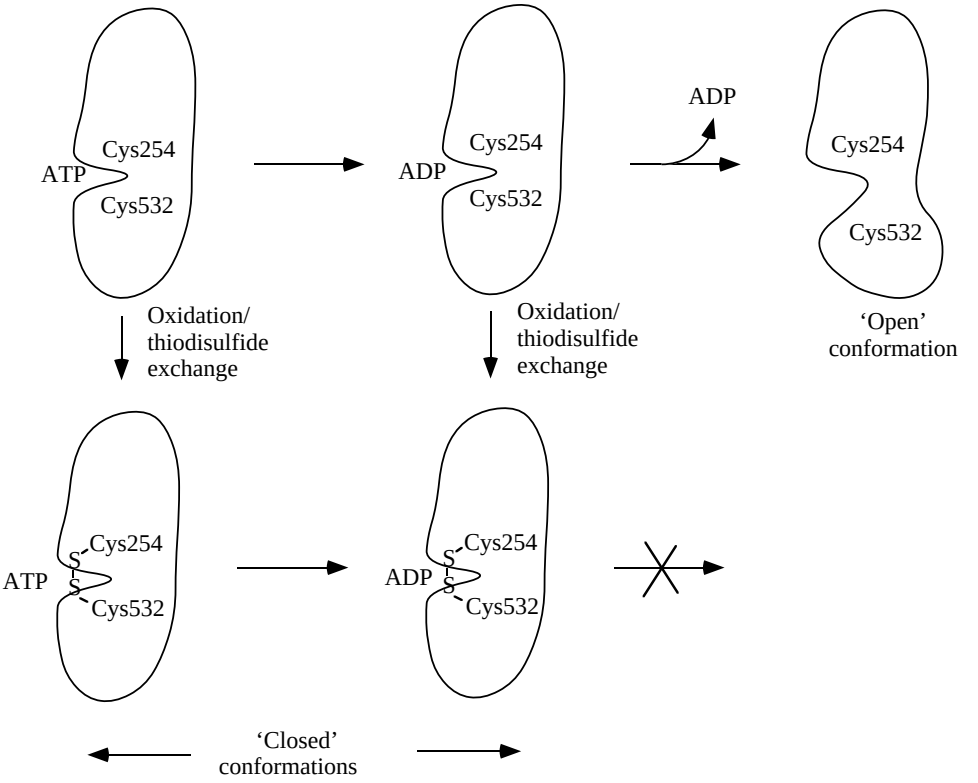

Fig. 2. Mechanism of inhibition of V-ATPase activity by disulfide bond formation. In the model shown, disulfide bond formation between Cys254 and Cys532 at the catalytic site on the V-ATPase A subunit sterically prevents the enzyme from adopting the 'open' conformation required for release of ADP and hence inhibits activity. For simplicity, only one of the three A subunits is shown.

below). Disulfide bond formation does not appear to inhibit nucleotide binding to the catalytic site, however, as indicated by 2-azido-[^{32}P]ATP labeling (Feng and Forgac, 1994). Instead, an alternative mechanism by which disulfide bond formation inhibits V-ATPase activity has been suggested (Forgac, 1998) and is illustrated in Fig. 2. 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 5′-adenylyl-imidodiphosphate (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 2 nm. Each β subunit is believed to cycle between these conformations during catalysis. Because Cys532 is located in the C-terminal domain of the V-ATPase A subunit, whereas Cys254 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 characterize further the catalytic nucleotide-binding site on the A subunit, site-directed mutagenesis of the yeast V-ATPase A subunit has also been carried out (Liu et al., 1997; MacLeod et al., 1998). It was shown that mutation of Glu286 to Gln leads to complete loss of activity (Liu et al., 1997), consistent with the postulated role for the corresponding glutamic acid residue in ATP hydrolysis by the F-ATPase β subunit (Weber and Senior, 1997). Similarly, mutation of the lysine in the glycine-rich loop sequence of the A subunit (Lys263) to glutamine also leads to loss of activity (Liu et al., 1997). In contrast, a variety of other mutations in the A subunit (including Gly250Asp in the glycine-rich loop and Cys284Val and Cys539Ser, representing two of the three conserved cysteine residues in the A subunit) lead to destabilization of the A subunit.

The effect of mutation of conserved aromatic residues in the C-terminal domain of the A subunit has also been investigated (MacLeod et al., 1998). Whereas the mutations Phe538Trp and Tyr532Ser caused significant changes in K_m and V_{max}, respectively, the largest effects on activity were observed for mutations at Phe452. The Phe452Ala mutant was completely inactive. These results suggest that all three of these aromatic residues may participate in the formation of the adenine-binding pocket on the A subunit. Finally, two residues on the B subunit (Tyr352 and Arg381), postulated to be contributed by the B subunit to the catalytic sites, have been mutagenized (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 participates 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 tritium-labeled 3′-*O*-(4-benzoyl)benzoyladenosine

5′-triphosphate ([^{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 between Ile164 and Asn171. 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. It has been shown that nucleotide binding to the catalytic site (as measured by 2-azido-[^{32}P]ATP modification) is not blocked by disulfide bond formation or by cystine modification of Cys254 but is blocked by reaction of Cys254 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, and nucleotide binding to this site is affected by changes occurring at the catalytic site, indicating that there is significant communication between the catalytic and noncatalytic sites on the enzyme.

To probe further 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 (Tyr370Ser, His180Gly and Asn181Val) caused only partial inhibition (activities ranged from 40 to 80 % of wild-type values). Similarly, mutation of A subunit residues predicted to be present at the noncatalytic sites (Phe479Ala and Arg483Gln) also led to only partial loss of activity (30–70 % of wild-type values) (MacLeod et al., 1998). Significantly, two A subunit mutations (Arg483Glu and Arg483Gln) led to a time-dependent increase in V-ATPase activity following addition of the enzyme 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

Although the V- and F-ATPases share amino acid sequence homology in the nucleotide-binding subunits of the peripheral domain as well as the proteolipid subunits of the integral domain (see below), no other subunits in the two complexes display such sequence homology. Nevertheless, two of the V_1 subunits (subunits D and E) are predicted to have a very high α-helical content (Bowman et al., 1995; Nelson et al., 1995), as is observed for the γ subunit of F_1 (Abrahams et al., 1994). The γ subunit plays a particularly important role in coupling of proton transport and ATP hydrolysis in the F-ATPase since it is the γ subunit that is induced to rotate within the $\alpha_3\beta_3$ hexamer during ATP hydrolysis (Noji et al., 1997; Cross and Duncan, 1996; Junge et al., 1996). Whether subunits D or E serve the function of the γ subunit in the V-ATPase complex remains to be determined.

Reassembly of the dissociated V_1 domain onto stripped coated vesicles containing the free V_o domain and restoration ATP-dependent proton transport has been demonstrated (Puopolo and Forgac, 1990), and this *in vitro* reassembly system has been used 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). Re-addition of this V_1(−C) subcomplex to stripped vesicles restores approximately 50 % of the control level of proton transport, although a significant amount of this activity may be due to residual subunit C attached to the V_o domain (T. Xu and M. Forgac, in preparation).

The purified V-ATPase complex has been reported to contain the 50 kDa subunit of the AP-2 adaptin complex (Myer and Forgac, 1993b), and treatment of the V-ATPase with cystine results in the removal of this protein and loss of V-ATPase activity (Liu et al., 1994). More recently, it has been found 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 (T. Xu and M. Forgac, in preparation). In yeast, subunit H is required for activity but not for assembly of the V-ATPase complex, and it is encoded by the *VMA13* gene (Stevens and Forgac, 1997). It therefore appears that subunit H, rather than AP50, is required for activity and stability of the reassembled coated-vesicle V-ATPase, although both subunit H and AP50 are removed upon cystine treatment (Liu et al., 1994). These results suggest that communication exists between subunit H and the catalytic nucleotide-binding site of the enzyme.

Structure and function of V_o 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 V-ATPases to DCCD (Arai et al., 1987a). This subunit has sequence homology to subunit c of F_o, from which it appears to have been derived by gene duplication 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 the fourth transmembrane segment (TM4) that is the site of DCCD reaction and is essential for proton transport. In contrast, the F-ATPase subunit c contains two transmembrane helices with the essential buried carboxyl group in TM2 (Fillingame, 1997). Subunit c appears to

contribute the same number of transmembrane helices (24) to the integral domain for both V- and F-ATPases, since there are six copies of this subunit in V_o (Arai et al., 1988) compared wth 12 copies in F_o (Fillingame, 1997). This means that there are half as many buried carboxyl groups in the V_o domain as in F_o, 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). In both cases, reaction of a single subunit c with DCCD is sufficient to block proton transport by the complex completely (Arai et al., 1987a).

Unlike the F_o domain, the V_o domain contains two additional proteolipid subunits, c′ and c″, both of which have homology to subunit c (Hirata et al., 1997). Subunit c′ also contains four transmembrane segments, with the buried carboxyl in TM4, whereas subunit c″ contains five transmembrane segments, with the buried carboxyl in TM3. Although 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_o 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_o subunits (a, d and c″) as well (Zhang et al., 1994).

Subunit a

Subunit a is a 100 kDa transmembrane glycoprotein (Adachi et al., 1990b) that contains an N-terminal hydrophilic domain and a C-terminal hydrophobic domain containing 6–9 putative transmembrane segments (Manolson et al., 1992). Reassembly and reconstitution studies have revealed that subunit a aids in proton conduction by the V_o 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 (Manolson et al., 1994). Thus, Vph1p is targeted to the vacuole while Stv1p is targeted to some other intracellular compartment, possibly endosomes or the Golgi apparatus. We have investigated the role of subunit a in proton transport and in 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 (Glu789, His743 and Lys593) led 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 of the F-ATPase a subunit, which plays a crucial role in proton conductance by F_o (Cain and Simoni, 1988). More recent studies have revealed that, although charged residues at positions 789 and 743 of Vph1p are not absolutely required for proton transport, both residues are in a position to influence activity significantly, as revealed by the altered pH-dependence of proton transport for all 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 affecting activity have been identified by random mutagenesis (Leng et al., 1998). Thus, mutation of either Leu739 or Leu746 to Ser led to almost complete loss of activity, possibly by turning the helix containing His743 away from the proton-conduction pathway. Similarly, mutation of His729 to Arg in the loop preceding the penultimate transmembrane segment caused inhibition of more than 90 % of proton-transport activity. In contrast, a cluster of five mutations in the region between Leu800 and Gly814 led to loss of assembly, suggesting that this region at the C terminus of the a subunit plays an important role in assembly of the V-ATPase complex (Leng et al., 1998).

Most recently, we have carried out analysis of the transmembrane topography of the yeast V-ATPase subunit a (Vph1p) using cysteine-scanning mutagenesis and labeling by sulfhydryl reagents of different membrane permeability, as well as proteolyic cleavage at engineered protease sites within subunit a (Leng et al., 1999). On the basis of these studies, we have proposed a model in which subunit a contains nine transmembrane helices, with the large N-terminal domain exposed on the cytoplasmic side of the membrane and the C-terminal region exposed on the luminal side of the membrane.

Subunit d

Subunit d is unique among the V_o subunits in possessing no putative transmembrane helices (Wang et al., 1988) but in remaining tightly bound to the V_o domain (Zhang et al., 1992), presumably through protein–protein interactions with other V_o 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.

Mechanism of ATP-dependent proton transport by the V-ATPases

Although the mechanism by which the V-ATPases carry out ATP-dependent proton transport has not been defined, it is likely that it will be similar to the mechanism proposed for coupling of proton transport and ATP hydrolysis by the F-ATPases (Cross and Duncan, 1996; Junge et al., 1996; Vik and Antonio, 1994). For the F-ATPases, hydrolysis of ATP by the β subunits is thought to drive rotation of the central γ subunit within the $\alpha_3\beta_3$ hexamer. Rotation of γ within F_1 has now been demonstrated using several techniques (Cross and Duncan, 1996; Junge et al., 1996), most recently by fluorescence microscopy (Noji et al., 1997). The γ subunit is thought to be held rigidly to the ring of c subunits (Ogilvie et al., 1997), such that rotation of γ causes rotation of the ring of c subunits relative to the adjacent a subunit. The a subunit is, in turn, held fixed relative to the $\alpha_3\beta_3$ hexamer by a secondary stalk consisting of the δ subunit and the soluble portions of the b

subunits. The a subunit provides access of protons to the buried carboxyl groups of subunit c through two hemi-channels that are displaced relative to each other (Vik and Antonio, 1994). As the ring of c subunits rotates, protons are picked up by the c subunit carboxyls from one hemi-channel, rotate through the bilayer, and are then released on the opposite side of the membrane through the second hemi-channel. Because rotation occurs in only one direction during ATP hydrolysis, and because the carboxyl groups must be protonated when in contact with the lipid bilayer and unprotonated when in contact with subunit a, this mechanism serves to convert the rotary motion induced by hydrolysis into a linear gradient of protons across the membrane.

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). For example, endosomes become progressively more acidic following receptor-mediated endocytosis, ensuring that ligand–receptor dissociation and receptor recycling occur from the correct compartment. Several mechanisms for the control of vacuolar acidification *in vivo* are discussed below.

Disulfide bond formation at the catalytic site

As described above, disulfide bond formation between Cys254 and Cys532 of the bovine V-ATPase subunit A leads to reversible inactivation of the V-ATPase in clathrin-coated vesicles (Feng and Forgac, 1992b, 1994). Moreover, 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 the 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 found that, even in the absence of reducing agents, the fraction of Cys254 in the reduced form increases with time, indicating that reduction of the Cys254–Cys532 disulfide bond comes about *via* thio-disulfide exchange involving a third subunit A 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 probably significantly higher. This is consistent with the observation that endocytotic 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). 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).

Yeast mutants have been used to conduct a preliminary test of the role of disulfide bond formation in the regulation of vacuolar pH in the Golgi apparatus (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 apparatus to the vacuole is observed (Stevens and Forgac, 1997). This defect 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 above), expression of a constitutively active V-ATPase in the Golgi apparatus might cause a similar missorting phenotype in yeast. The Cys261Val 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. Although 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 result suggests that disulfide bond formation may not be regulating V-ATPase activity in the Golgi apparatus, at least in yeast. Alternatively, vacuolar acidification may be playing a somewhat different role in targeting in yeast and mammalian cells. Analysis of *CYS4* mutants that show altered redox potential as a result of a defect in cysteine biosynthesis suggests that disulfide bond formation can play a role in regulating V-ATPase activity *in vivo* (Oluwatosin and Kane, 1997).

Disulfide bond formation at the catalytic site of the V-ATPase has recently been shown to be induced by the nitric-oxide-generating reagent S-nitrosoglutathione (SNG) (Forgac, 1999). Although the concentrations of SNG required for inhibition are relatively high ($K_{0.5}$=200–400 μmol l^{-1}), suggesting that this mechanism 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 affect 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 inactivating V-ATPases in the corresponding membranes. 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 thio-disulfide bond rearrangement, as originally postulated (Feng and Forgac, 1994).

Reversible association and dissociation of V_1 and V_o

Kane (1995) has demonstrated that, in yeast, glucose deprivation results in a rapid dissociation of V_1 and V_o domains, and that this effect is reversed upon readdition of glucose. These results suggest 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 (Sumner et al., 1995) and starvation (Graf et al., 1996) in insects.

Free V_1 domains have also been shown to exist in the cytoplasm of MDBK cells (Myers and Forgac, 1993a). A significant population of free V_o domains is also observed in clathrin-coated vesicle membranes (Zhang et al., 1992). Because free V_1 domains are not active in hydrolysis of MgATP (Puopolo et al., 1992b) and free V_o domains are not normally functional as passive proton channels (Zhang et al., 1992), the existence of significant populations of these free complexes in the cell does not compromise energy stores or proton gradients across vacuolar membranes. 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

A variety of conditions cause a change in the efficiency of coupling between 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_o 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 activity despite the continued presence of 50 % of control levels of ATP hydrolysis (Adachi et al., 1990a). Recently, sodium azide has been shown to inhibit completely proton transport by the V-ATPase without affecting 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 alter the coupling between proton transport and ATP hydrolysis and that this change in coupling may represent an important mechanism of controlling vacuolar acidification *in vivo*. The normal intracellular signals that affect this coupling efficiency, however, remain to be identified.

Changes in Cl^- channel activity

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). In coated vesicles, this dissipation of the membrane potential is accomplished by a Cl^- channel that is distinct from the V-ATPase (Arai et al., 1989). Moreover, the activity of this Cl^- channel is regulated by phosphorylation by cyclic-AMP-dependent protein kinase (Mulberg et al., 1991). Thus, dephosphorylation of the Cl^- channel decreased Cl^- conductance and ATP-dependent acidification, while phosphorylation by protein kinase A increased both activities. These results suggest that changes in the activity of a Cl^- channel required for vacuolar acidification represent an important mechanism by which the pH of intracellular compartments can be controlled.

Conclusions

The V-ATPases are an essential family of ATP-dependent proton pumps that play a wide variety of roles in eukaryotic cells, both within intracellular compartments and at the cell surface. The V-ATPases in clathrin-coated vesicles are involved in such processes as receptor-mediated endocytosis, intracellular membrane trafficking and neurotransmitter uptake, while those in yeast function in protein degradation and coupled transport. Using techniques of protein biochemistry and mutagenesis, considerable progress has been made in understanding the structure of the V-ATPases and the role of individual subunits and residues in ATP-dependent proton transport. Because of the diversity of functions served by the V-ATPases in cells, it is likely that the mechanisms involved in the regulation of vacuolar acidification will turn out to be similarly complex.

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