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# Structure and Regulation of the V-ATPases

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The V-ATPases are ATP-dependent proton pumps present in both intracellular compartments and the plasma membrane. They function in such processes as membrane traffic, protein degradation, renal acidification, bone resorption and tumor metastasis. The V-ATPases are composed of a peripheral $V_1$ domain responsible for ATP hydrolysis and an integral $V_0$ domain that carries out proton transport. Our recent work has focused on structural analysis of the V-ATPase complex using both cysteine-mediated cross-linking and electron microscopy. For cross-linking studies, unique cysteine residues were introduced into structurally defined sites within the B and C subunits and used as points of attachment for the photoactivated cross-linking reagent MBP. Disulfide mediated cross-linking has also been used to define helical contact surfaces between subunits within the integral $V_0$ domain. With respect to regulation of V-ATPase activity, we have investigated the role that intracellular environment, luminal pH and a unique domain of the catalytic A subunit play in controlling reversible dissociation in vivo.

**KEY WORDS:** vacuolar ($H^+$)-ATPase; membrane protein structure; pH regulation; yeast.

## INTRODUCTION

The vacuolar ($H^+$)-ATPases (or V-ATPases) are ATP-dependent proton pumps present in both intracellular and plasma membranes (Nishi and Forgac, 2002). Intracellular V-ATPases function in such processes as receptor recycling following receptor-mediated endocytosis, intracellular targeting of newly synthesized lysosomal enzymes, protein processing and degradation and the coupled transport of small molecules, such as neurotransmitters. They also function in infection of cells by certain envelope viruses, such as influenza virus, and the killing of cells by certain bacterial toxins, such as diphtheria and anthrax toxin (Abrami *et al*., 2004). Plasma membrane V-ATPases also function in a variety of normal and disease processes, including renal acidification, bone resorption, pH homeostasis, sperm maturation, coupled transport, and tumor metastasis (Sennoune *et al*., 2004). V-ATPases have therefore been identified as potential therapeutic targets in treating such diseases as osteoporosis and cancer. This review will focus primarily on recent work from our laboratory aimed at elucidating the structure and regulation of the V-ATPases.

## STRUCTURE OF THE V-ATPases

The V-ATPases are composed of two domains (Nishi and Forgac, 2002) (Fig. 1). The $V_1$ domain is a 600–650 kDa peripheral complex composed of eight different subunits (subunits A–H) of molecular mass 70–13 kDa that are present in a stoichiometry of $A_3B_3C_1D_1E_1F_1G_2H_2$ (Arai *et al*., 1988; Xu *et al*., 1999). The $V_1$ domain is responsible for ATP hydrolysis, and both the 70-kDa A subunits and the 60-kDa B subunits participate in nucleotide binding (MacLeod *et al*., 1998, 1999; Vasilyeva *et al*., 2000). The nucleotide binding sites are actually at the interface of the A and B subunits, with the three catalytic sites located primarily on the A subunits and three "noncatalytic" sites located principally on the

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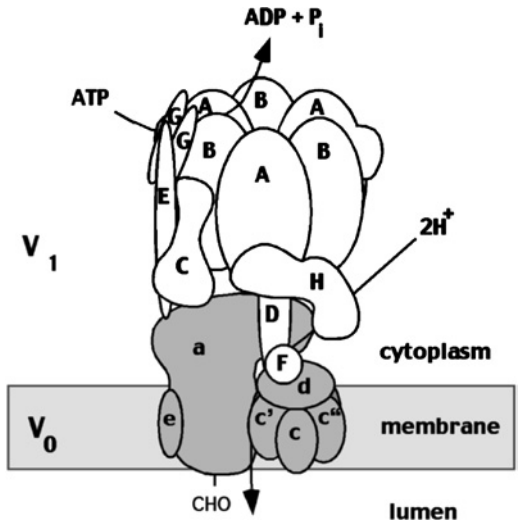


**Fig. 1.** Structural model of the yeast V-ATPase. The peripheral $V_1$ domain responsible for ATP hydrolysis is shown in white while the integral $V_0$ domain that carries out proton transport is shaded. Subunit arrangement is based in part on recent studies employing cysteine-mediated cross-linking and electron microscopy (Arata *et al*., 2002a,b; Wilkens *et al*., 2004; Inoue and Forgac, 2005). Knobs on catalytic A subunits correspond to the "non-homologous" region (Zhang *et al*., 2003). V-ATPases operate by a rotary mechanism (Imamura *et al*., 2003; Hirata *et al*., 2003). ATP hydrolysis drives rotation of a rotor domain, containing subunits D and F in $V_1$ attached to the proteolipid ring (subunits c, c′ and c″) and subunit d of $V_0$, with rotation occurring relative to the stator domain, that includes the $A_3B_3$ catalytic head of $V_1$ and subunit a of $V_0$ attached via a peripheral stalk composed of subunits C, E, G, H, and the hydrophilic domain of subunit a. It is movement of the proteolipid ring relative to subunit a that drives proton transport (see text).

B subunits. Although the function of the noncatalytic nucleotide binding sites has not been established, they have been proposed to serve a regulatory role (MacLeod *et al*., 1998).

The $V_0$ domain is a 260 kDa integral complex composed of six different subunits. In yeast, these subunits are a, c, c′, c″, d, and e and have molecular masses 100–9 kDa (Sambade and Kane, 2004). They are present in a stoichiometry of $a_1d_1e_xc_{4-5}c'_1c''_1$ (Arai *et al*., 1988; Powell *et al*., 2000). In animal cells, subunit c′ is absent but an additional glycoprotein of 45 kDa (termed Ac45) is present (Supek *et al*., 1994). The $V_0$ domain is responsible for proton translocation, with both the proteolipid subunits (c, c′, and c″) (Hirata *et al*., 1997) and subunit a containing residues essential for proton transport (Leng *et al*., 1996, 1998; Kawasaki-Nishi *et al*., 2001b).

The V-ATPases thus resemble the F-ATPases (or ATP synthases) of mitochondria, chloroplasts, and bacteria, which normally function in ATP synthesis (Cross and Mueller, 2004; Weber and Senior, 2003, Fillingame *et al*., 2002; Yoshida *et al*., 2001). In fact the two classes of ATPase share sequence homology in both the nucleotide binding subunits (with A homologous to the F-ATPase $\beta$ and B homologous to the F-ATPase $\alpha$ (Zimniak *et al*., 1988; Bowman *et al*., 1988)) as well as in the proteolipid c subunits (Mandel *et al*., 1988). Electron microscopy has revealed that, like the F-ATPases, the V-ATPases are composed of a head domain ($V_1$) and a membrane domain ($V_0$) connected by both a central stalk and a peripheral stalk (Wilkens *et al*., 1999; Boekema *et al*., 1999). As discussed below, the central and peripheral stalks play an important role in the rotary mechanism by which both the V and F-ATPases operate. Nevertheless, the V-ATPases show considerably greater structural complexity than the F-ATPases, including the presence of more than one peripheral stalk (Boekema *et al*., 1999; Wilkens *et al*., 2004).

## SUBUNIT DISTRIBUTION BETWEEN CENTRAL AND PERIPHERAL STALKS

To elucidate the arrangement of subunits in the V-ATPase complex, we have taken advantage of the homology between the nucleotide binding subunits of the V and F-ATPase and the X-ray crystal structure of $F_1$ (Abrahams *et al*., 1994) to create a molecular model of the $A_3B_3$ head domain of the V-ATPase (MacLeod *et al*., 1998)). We have shown that this model accurately predicts the identity of residues at both the catalytic and noncatalytic nucleotide binding sites on the A and B subunits (MacLeod *et al*., 1998, 1999; Vasilyeva *et al*., 2000). This has allowed us to use this model of the B subunit as a sort of "molecular scaffold." We have introduced unique cysteine residues into a cys-less form of subunit B and used these cysteine residues as sites of attachment for the photoreactive cross-linking reagent maleimidobenzophenone (MBP). MBP reacts with the structurally defined, unique cysteine residue in the B subunit via the maleimide moiety and then, following UV irradiation, is able to cross-link to other residues within approximately 10 Å of the initial site on the B subunit. It should be noted that the photoreactive species generated is not specific for a particular residue, but is able to cross-link to a wide variety of sites in nearby subunits. The cross-linked products are then analyzed by SDS-PAGE and Western blot using subunit-specific antibodies to identify those subunits that are proximal to specific sites in subunit B.

Using this approach, we have shown that subunits E, G, and H are able to crosslink to sites on the B subunit facing the exterior of the complex (Arata *et al*., 2002a,b; Wilkens *et al*., 2004), indicating that these subunits are part of the peripheral stalk (or stator) connecting the $V_1$ and $V_0$ domains. Interestingly, subunit G shows

cross-linking to only sites near the top of the B subunit, furthest from the membrane (Arata *et al*., 2002b), whereas subunit H shows cross-linking to sites closest to the interface of $V_1$ and $V_0$ (Wilkens *et al*., 2004). Subunit E, on the other hand, shows cross-linking to sites extending the entire length of the B subunit, indicating that subunit E exists in a very extended conformation on the exterior of the complex (Arata *et al*., 2002a,b). By contrast, subunit D shows cross-linking to only residues facing the interior of the $A_3B_3$ hexamer, indicating that subunit D forms part of the central (or rotary) stalk connecting $V_1$ and $V_0$ (Arata *et al*., 2002b). Difference mapping of subunit H-containing and subunit H-depleted complexes of the bovine-coated vesicle V-ATPase have also placed subunit H at the interface of the $V_1$ and $V_0$ domains (Wilkens *et al*., 2004).

We have now extended these studies (Inoue and Forgac, 2005) using the recently published X-ray crystal structure of subunit C. Subunit C is a 40-kDa protein that contains two globular domains: a "foot" domain containing both the amino and carboxyl-terminal portions of the protein and a "head" domain containing the central region of the sequence (Drory *et al*., 2004). These two domains are connected by a short alpha-helical neck. We have shown that the head domain of subunit C is able to cross-link to both subunits G and E whereas the foot domain shows cross-linking to subunits E and a (Inoue and Forgac, 2005). These results confirm the earlier suggestion that subunit E exists in a very extended conformation on the outer surface of the complex and place subunit C at the interface of the $V_1$ and $V_0$ domains. Moreover, subunit C is oriented such that the head domain is facing away from the membrane and the foot domain directly interacts with subunit a in the $V_0$ domain. This places subunit C in an ideal position to regulate interactions between the $V_1$ and $V_0$ domains, a role it has been proposed to fill in controlling in vivo dissociation of the V-ATPase complex (see "Regulation of V-ATPase Activity" below).

## SUBUNIT ARRANGEMENT AND FUNCTION WITHIN THE $V_0$ DOMAIN

Of the six subunits present in the yeast $V_0$ domain, three (c, c′ and c″) are highly hydrophobic proteolipid subunits with sequence homology to the c subunit of the F-ATPases (Mandel *et al*., 1988; Hirata *et al*., 1997). Subunits c and c′ are 16 kDa in size and each contains four transmembrane helices, with both the N and C-termini present on the luminal side of the membrane (Nishi *et al*., 2001). Subunit c″ is 23 kDa and contains either four or five transmembrane helices (Nishi *et al*., 2001, 2003; Flannery *et al*., 2004). There is disagreement about whether the first hydrophobic segment does or does not cross the membrane, but there is consensus that it is not required for function. Each proteolipid subunit contains a single buried glutamic acid residue that is essential for function. For subunits c and c′, this essential acidic residue is present in TM4. Subunit c″ contains two buried glutamate residues, one in TM4 and one in TM2 (assuming a four TM model). Only the glutamate in TM2 is essential for proton transport (Hirata *et al*., 1997). The three proteolipid subunits form a ring with the stoichiometry $c_{4-5}c'_1c''_1$ (Arai *et al*., 1988; Powell *et al*., 2000), and containing between 24 and 29 transmembrane helices. This is larger than the $F_0$ c ring of *E. coli* and yeast mitochondria, which each contain 10 copies of the two-membrane spanning c subunit (giving a structure containing 20 transmembrane helices) (Jiang *et al*., 2001; Stock *et al*., 1999), but smaller than the c ring of the $Na^+$ transporting ATPase of *Enterococcus*, which, from the X-ray structure, has 10 copies of a 4 TM c subunit giving 40 transmembrane helices (Murata *et al*., 2005). It is interesting to note, however, that the *Enterococcus* c subunit also apparently forms a seven-membered ring containing 28 transmembrane helices, as revealed by electron microscopy (Murata *et al*., 2003), a structure much closer in size to that of the eukaryotic V-ATPase.

In addition to the proteolipid subunits, the yeast $V_0$ domain also contains a 10-kDa hydrophobic protein (subunit e) of unknown function (Sambade and Kane, 2004), a 32-kDa hydrophilic protein (subunit d) that appears to sit between the proteolipid ring and the central stalk of $V_1$ (Iwata *et al*., 2004), and a 100-kDa integral membrane protein designated subunit a. Subunit a contains an amino terminal hydrophilic domain oriented towards the cytoplasmic side of the membrane and a hydrophobic carboxyl-terminal domain containing nine transmembrane helices (Leng *et al*., 1999). The C-terminal domain contains several buried charged residues that appear to function in proton transport (Leng *et al*., 1996, 1998; Kawasaki-Nishi *et al*., 2001b). In particular, Arg735 in TM7 is absolutely required for proton translocation (Kawasaki-Nishi *et al*., 2001b).

Subunit a is thought to serve two roles in proton transport. First, it is hypothesized to provide aqueous access channels that allow protons to reach the buried carboxyl groups on the proteolipid ring from the cytoplasmic side and to exit these sites to the luminal side of the membrane (Vik *et al*., 2000). It is as part of these aqueous hemichannels that residues such as Glu789, His729 and Arg799 are thought to function (Leng *et al*., 1996, 1998; Kawasaki-Nishi *et al*., 2001b). Second, the arginine residue at position 735 is thought to directly interact with the buried carboxyl groups on the proteolipid ring, thereby displacing

the proton into the luminal hemichannel and stabilizing the carboxyl group in its charged form. Each c subunit thus picks up a proton from the cytoplasmic hemichannel, the c ring rotates and the protonated c subunit releases its proton into the luminal hemichannel upon interaction with the critical arginine residue (Nishi and Forgac, 2002). Rotation of the proteolipid ring is driven by ATP-dependent rotation of the central stalk of the $V_1$ domain, to which it is connected. Rotation of both the central stalk and the proteolipid ring have now been demonstrated for the V-ATPases (Imamura *et al*., 2003; Hirata *et al*., 2003), as was previously demonstrated for the F-ATPases (Duncan *et al*., 1995; Noji *et al*., 1997; Sambongi *et al*., 1999).

Disulfide-mediated cross-linking studies between cysteine residues introduced into transmembrane helices of subunits a, c′ and c″ have demonstrated that TM7 of subunit a containing the critical Arg735 is in close proximity (within several angstroms) of TM4 of subunit c′ and TM2 of subunit c″ (Kawasaki-Nishi *et al*., 2003; Wang *et al*., 2004). Each of these helices contains the essential buried glutamate residue that undergoes reversible protonation and deprotonation during proton transport. Moreover, the cross-linking results suggest that these helices in both subunit a and in the proteolipid subunits undergo rotation relative to the surrounding helices (Kawasaki-Nishi *et al*., 2003; Wang *et al*., 2004). This helical rotation may assist in the opening and closing of the aqueous hemichannels that control proton access, and may be coupled to rotation to the proteolipid ring as a whole. A similar helical rotation has been documented within the $F_0$ domain (Jiang and Fillingame, 1998) and has been supported by the available NMR structures of subunit c (Girvin *et al*., 1998; Rastogi and Girvin, 1999).

## REGULATION OF V-ATPASE ACTIVITY IN VIVO

Several mechanisms have been proposed to be involved in regulation of V-ATPase activity under in vivo conditions. These include reversible disulfide-bond formation between conserved cysteine residues at the catalytic site on the A subunit (Feng and Forgac, 1992, 1994), changes in coupling efficiency between ATP hydrolysis and proton transport (Shao *et al*., 2003; Shao and Forgac, 2004), changes in distribution of V-ATPases between different cellular membranes (Brown and Breton, 2000), and reversible changes in assembly of the $V_1$ and $V_0$ domains (Kane, 1995). With respect to the intracellular distribution of V-ATPases, targeting of V-ATPases to different cellular membranes is controlled by isoforms of subunit a. In mammalian cells, subunit a exists in four different isoforms (a1–a4), with a3 and a4 responsible for targeting the V-ATPase to the plasma membrane of osteoclasts and renal intercalated cells, respectively (Nishi and Forgac, 2000; Toyomura *et al*., 2000; Oka *et al*., 2001). In yeast two isoforms of subunit a exist (Vph1p and Stv1p) (Manolson *et al*., 1992, 1994), which target the V-ATPase to the vacuole and a late Golgi compartment, respectively (Kawasaki-Nishi *et al*., 2002). Chimera analysis has demonstrated that targeting information is located in the hydrophilic amino-terminal domain (Kawasaki-Nishi *et al*., 2002). We have shown that V-ATPase complexes containing different isoforms of subunit a differ not only in intracellular distribution but also in their degree of assembly and in the tightness of coupling of proton transport and ATP hydrolysis (Kawasaki-Nishi *et al*., 2001a). Thus, Vph1p-containing complexes show a 10-fold greater assembly and a four- to fivefold tighter coupling than Stv1p-containing complexes, which may help to explain the lower pH of the vacuole relative to the Golgi.

The tightness of coupling also appears to be controlled in part by a novel domain of the catalytic A subunit termed the non-homologous region (Shao *et al*., 2003). This 90 amino acid domain is present and conserved in all V-ATPase sequences but is absent from the homologous $\beta$ subunit of the F-ATPases (Zimniak *et al*., 1988). Mutations in this region have been shown to either increase or decrease the coupling efficiency of the V-ATPase, depending upon the site of the mutation (Shao *et al*., 2003). The results suggest that the wild-type enzyme is not optimally coupled, but exists in a state where the coupling efficiency can be either increased or decreased, depending upon the needs of the cell.

Reversible dissociation of the V-ATPase has been shown to play an important role in regulation of activity in yeast (Kane, 1995), insect cells (Sumner *et al*., 1995) and, most recently, in mammalian cells (Trombetta *et al*., 2003). In yeast, reversible dissociation occurs in response to glucose depletion, occurs rapidly and without the need for new protein synthesis, and does not involve many of the signal transduction pathways known to be altered in response to nutrient deprivation (Parra and Kane, 1998). Dissociation also requires a catalytically active enzyme (Parra and Kane, 1998; MacLeod *et al*., 1999). In addition. dissociation and reassembly appear to be independently controlled processes. Thus, dissociation (but not reassembly) requires the presence of an intact microtubular network (Xu and Forgac, 2001), whereas reassembly (but not dissociation) requires a novel complex termed RAVE, that includes the ubiquitin ligase component Skp1p (Seol *et al*., 2001; Smardon *et al*., 2002).

We have shown that in vivo dissociation is critically dependent upon the membrane environment in which the V-ATPase resides. Thus, V-ATPases containing Vph1p

that are targeted to the vacuole undergo dissociation whereas complexes containing Stv1p that reside in the Golgi do not (Kawasaki-Nishi *et al*., 2001a). If Stv1p complexes are redirected to the vacuole, dissociation is then observed. Moreover, if Vph1p containing complexes are prevented from reaching the vacuole by disruption of normal vacuolar targeting, dissociation is less complete than is observed for complexes residing in the vacuole (Kawasaki-Nishi *et al*., 2001a). We hypothesized that the luminal acidic pH may play a role in controlling dissociation of the V-ATPase, since if the luminal pH becomes too alkaline, the cell may prevent dissociation to avoid compartments becoming even further alkalinized. This hypothesis was confirmed by the observation that neutralization of the vacuole with the weak base chloroquine effectively blocked dissociation without inhibition of activity (Shao and Forgac, 2004). The nature of the pH sensor responsible for sensing the luminal pH and conveying this information to the remainder of the complex remains uncertain, but subunit a is a possible candidate.

We have also identified mutations in the non-homlogous domain of subunit A that block dissociation without inhibition of activity, suggesting a role for this domain in controlling dissociation (Shao *et al*., 2003). Interestingly, the isolated non-homologous domain is able to bind to the $V_0$ domain in the absence of the remainder of the $V_1$ complex, and this association is disrupted in response to glucose depletion, suggesting that interaction between $V_0$ and the non-homologous domain may play an important role in triggering release of $V_1$ from $V_0$ (Shao and Forgac, 2004). Further work will be required to elucidate the details of how reversible dissociation of the V-ATPase is regulated in vivo.

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