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Structure and Properties of the Coated Vesicle (H^+)-ATPase

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Clathrin-coated vesicles play an important role in both receptor-mediated endocytosis and intracellular membrane traffic in eukaryotic cells. The coated vesicle (H^+)-ATPase functions to provide the acidic environment within endosomes and other intracellular compartments necessary for receptor recycling and intracellular membrane traffic. The coated vesicle (H^+)-ATPase is composed of nine different subunits which are divided into two distinct domains. The peripheral V_1 domain, which has the structure $73_3:58_3:40_1:34_1:33_1$, possesses the nucleotide binding sites of the (H^+)-ATPase. The integral V_0 domain, which has the composition $100_1:38_1:19_1:17_6$, contains the pathway for proton conduction across the membrane. Topographical analysis indicates a structure for the coated vesicle (H^+)-ATPase very similar to that of the F-type ATPases. Reassembly studies have allowed us to probe the function of particular subunits in this complex and the activity properties of the separate domains. These studies have led to insights into possible mechanisms of regulating vacuolar acidification.

KEY WORDS: Coated vesicle proton pump; V-ATPase structure; subunit function; catalytic residues; regulation of vacuolar acidification.

1. FUNCTION OF THE COATED VESICLE (H^+)-ATPASE

1.1. Function in Receptor-Mediated Endocytosis

Clathrin-coated vesicles play an important role in both receptor-mediated endocytosis and intracellular membrane traffic (for review, see Forgac, 1989). In the endocytic pathway, clathrin-coated pits provide the site at which a wide varity of cell surface receptors (together with their bound ligands) become concentrated. These include receptors for the cholesterol carrier LDL [low-density lipoprotein (Brown and Goldstein, 1986)], the iron-carrying protein transferin (Chievhanover *et al.*, 1983), asialoglycoproteins (Ashwell and Harford, 1982), immunoglobins [such as polyIgA (Mostov *et al.*, 1984)], and various hormones and growth factors, such as insulin and EGF (epidermal growth factor (Pastan and Willingham, 1981)). Following their concentration in coated pits, these ligand-receptor complexes enter the cell upon pinching off of the coated pit to form a clathrin-coated vesicle (Pearse, 1976). Rapid uncoating of the vesicle and subsequent membrane fusion events lead to delivery of the ligand-receptor complexes to an endosomal compartment referred to as CURL (compartment of uncoupling of receptor and ligand (Geuze *et al.*, 1983). Acidification of CURL by the vacuolar (H^+)-ATPases causes dissociation of internalized ligands from their receptors and allows the unoccupied receptors to recycle to the cell surface where they again become available to bind ligand. The dissociated ligands within the lumen of CURL are then free to continue their movement toward their final cellular destination, which in many cases is the lysosome, where they are degraded.

1.2. Function in Intracellular Membrane Traffic

Clathrin-coated vesicles play an analogous role in the intracellular targeting of newly synthesized lysosomal enzymes from the Golgi to lysosomes (Creek and

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Sly, 1984). During their transit of the Golgi, lysosomal enzymes become tagged with mannose-6-phosphate (Man-6-P) which allows them to bind to the Man-6-P receptor located in the trans-Golgi compartment. Within the trans-Golgi, Man-6-P receptors, together with their bound lysosomal enzymes, become concentrated in clathrin-coated pits which pinch off to form clathrin-coated vesicles. Vesicle uncoating and delivery to an acidic compartment results in dissociation of lysosomal enzymes from Man-6-P receptors and recycling of the receptors to the trans-Golgi where they are available for multiple rounds of binding. The free lysosomal enzymes are then targeted for delivery to lysosomes.

1.3. Acidification of Coated Vesicles *In Vivo*

Acidification by the vacuolar (H^+)-ATPases thus provides the criticial signal which activates dissociation of ligands from their receptors. We (Forgac *et al.*, 1983) and others (Stone *et al.*, 1983; van Dyke *et al.*, 1984) have demonstrated that clathrin-coated vesicles contain a (H^+)-ATPase capable of acidifying the lumen of the vesicle, and endosomes have been shown to acidify by a similar mechanism (Galloway *et al.*, 1983; Yamashiro *et al.*, 1983). Nevertheless, considerable data suggest that during the course of endocytosis, significant acidification of the endosome does not occur until delivery of ligand–receptor complexes to CURL. Thus, no dissociation of ligand–receptor complexes was observed in endocytic coated vesicles and small peripheral endosomes (Geuze *et al.*, 1983). In addition, based on their failure to accumulate DAMP (a weak base which can be visualized in the electron microscope), endocytic coated vesicles appeared to be neutral organelles within the cell (Anderson and Orci, 1988). Finally, rat liver endocytic coated vesicles loaded with the pH-sensitive fluorescence probe FITC-dextran by perfusion were shown to be incapable of ATP-dependent acidification (Fuchs *et al.*, 1987).

To address whether the failure of endocytic coated vesicles to acidify was due to the absence of the proton pump, we have carried out immunocytochemical studies of MDBK cells, a bovine epithelial cell line, using monoclonal antibodies directed against the coated vesicle (H^+)-ATPase (Marquez-Sterling *et al.*, 1991). We have demonstrated that, in addition to staining endosomes, lysosomes, and portions of the Golgi, these antibodies label the plasma membrane and vesicles just beneath the plasma membrane. Moreover, significant colocalization of the (H^+)-ATPase with clathrin was observed at both the cell surface and in these peripheral vesicles, indicating that the vacuolar proton pump is indeed present in endocytic coated vesicles (Marquez-Sterling *et al.*, 1991). These results, taken together with those described above, suggest that endocytic coated vesicles are not acidified because the activity of the coated vesicle (H^+)-ATPase is suppressed within the cell. Possible mechanisms of regulating vacuolar acidification will be discussed in Sec. 6.

1.4. Function of the Coated Vesicle (H^+)-ATPase in Brain

In addition to the functions discussed above, clathrin-coated vesicles also play a significant role at synaptic terminals where they are involved in membrane retrieval following synaptic vesicle fusion at the presynaptic membrane (Pfeffer and Kelly, 1985). Because synaptic vesicles contain a vacuolar (H^+)-ATPase in order to provide the electrochemical driving force for uptake of neurotransmitters (Cidon and Sihra, 1989), it is likely that the (H^+)-ATPase in brain coated vesicles may be enroute from the synaptic terminal to synaptic vesicles. If this is the case, it will be of great interest to determine whether the (H^+)-ATPase is constitutively active throughout its transit of the plasma membrane and these intracellular compartments or, as appears to be the case in other cell types, the activity of the pump is suppressed until acidification is required in synaptic vesicles.

2. ACTIVITY PROPERTIES OF THE COATED VESICLE (H^+)-ATPASE

2.1. Ion Transport Properties

The coated vesicle (H^+)-ATPase transports protons in a unidirectional manner from the cytoplasmic side of the membrane to the lumenal side, uncoupled to the countertransport of another cation (Forgac and Cantley, 1984). As a result, the coated vesicle (H^+)-ATPase is electrogenic and requires the movement of a compensating charge for any significant proton transport to occur. The membrane potential generated by the proton pump is dissipated by a parallel chloride conductance which is catalyzed by a chloride channel present in coated vesicles (Arai *et al.*, 1989). This chloride channel is separable from the (H^+)-ATPase and has been partially purified and reconstituted

(Mulberg *et al.*, 1991; Xie *et al.*, 1989). Because of the dependence of proton transport on chloride conductance, regulation of chloride channel activity provides an important potential mechanism for controlling vacuolar acidification (see Sec. 6).

2.2. Inhibitor Specificity

The coated vesicle (H^+)-ATPase, like other vacuolar (H^+)-ATPases (Forgac, 1989), is sensitive to micromolar concentrations of NEM and NBD-Cl (Arai *et al.*, 1987b). In both cases this inhibition is blocked in the presence of ATP, suggesting that both reagents react with groups present at the catalytic site of the enzyme. We have recently identified the catalytic cysteine residue responsible for the NEM-sensitivity of the coated vesicle (H^+)-ATPase (Feng and Forgac, 1992) (see Sec. 4.2). In addition, we have demonstrated that both NEM and NBD-Cl inhibit activity of the coated vesicle (H^+)-ATPase through reaction with the same group. Thus, reaction of the enzyme with NBD-Cl followed by extensive reaction with NEM and reversal of the NBD-Cl modification with DTT results in almost full restoration of activity.

Unlike the P-type ATPases (Pedersen and Carafoli, 1987a,b), the coated vesicle (H^+)-ATPase is resistant to vanadate (Forgac *et al.*, 1983), consistent with the absence of a phosphorylated intermediate during turnover of this enzyme (Forgac and Cantley, 1984). In addition, the coated vesicle (H^+)-ATPase can be distinguished from the F-type ATPases by its resistance to oligomycin and aurovertin (Forgac *et al.*, 1983). Nevertheless, the coated vesicle pump is sensitive to 50 μM DCCD, a carboxyl reagent which inhibits both proton transport and, for the membrane-bound enzyme, ATPase activity (Arai *et al.*, 1987a). Inhibition by duramycin has also been observed, but its effect on acidification is complicated by the fact that it also inhibits chloride channel activity (Stone *et al.*, 1984).

The most specific inhibitor yet identified for the vacuolar (H^+)-ATPases is bafilomycin (Bowman E. J. *et al.*, 1988a) (also see p. 361). As with other vacuolar (H^+)-ATPases, bafilomycin inhibits the coated vesicle (H^+)-ATPase at nanomolar concentrations (unpublished observations).

2.3. Nucleotide Binding Sites

Evidence for multiple nucleotide binding sites on the coated vesicle (H^+)-ATPase has come from several sources. The dependence of ATP hydrolysis by the purified enzyme on the concentration of ATP indicates the existence of two ATP binding sites with K_d values of 80 and 800 μM (Arai *et al.*, 1989). Interestingly, while proton transport increases on saturation of the higher-affinity site, it decreases as the low-affinity site is occupied, such that the H^+/ATP stoichiometry decreases with increasing ATP concentration. A still higher-affinity site, with a K_d value of approximately 200 nM, has been identified as the site which activates dissociation of the peripheral V_1 domain from the integral V_0 domain (Arai *et al.*, 1989) (see Sec. 3.2). Occupation of this site thus loosens the structure of the coated vesicle (H^+)-ATPase.

Further evidence for the existence of multiple nucleotide binding sites has come from studies of the nucleotide analog TNP-ATP (Adachi *et al.*, 1990a). TNP-ATP inhibits activity of the purified, reconstituted (H^+)-ATPase by binding to two sites with K_d values of 50 nM and 3 μM. This analog is also able to protect both the 73-kDa A subunit and the 58-kDa B subunit of the (H^+)-ATPase from typtic cleavage, although the site responsible for this protection has a much lower affinity (K_d approximately 1–2 mM) (Adachi *et al.*, 1990a).

The final evidence for the existence of multiple nucleotide binding sites in the (H^+)-ATPase complex is the existence of multiple copies of two subunits (the A and B sununits) which have, either through direct chemical labeling studies or through sequence homology, been shown to possess nucleotide binding sites (see Sec. 4.1). The ATP sites responsible for the diverse effects of ATP on the activity and structure of the (H^+)-ATPase discussed above have yet to be definitively assigned to either the A or B subunits.

3. STRUCTURE OF THE COATED VESICLE (H^+)-ATPASE

3.1. Structural Model

Our current model of the coated vesicle (H^+)-ATPase (Adachi *et al.*, 1990b), based on the structural data described below, is shown in Fig. 1. The (H^+)-ATPase complex is divided into a peripheral V_1 domain containing subunits of molecular mass 73(A), 58(B), 40, 34, and 33 kDa and an integral V_0 domain comprised of the 100-, 38-, 19-, and 17(c)-kDa subunits. The entire complex, which has a molecular weight of 750,000, contains three copies each of the A and B sununits, six copies of the c subunit, and one copy of the remaining polypeptides. The catalytic

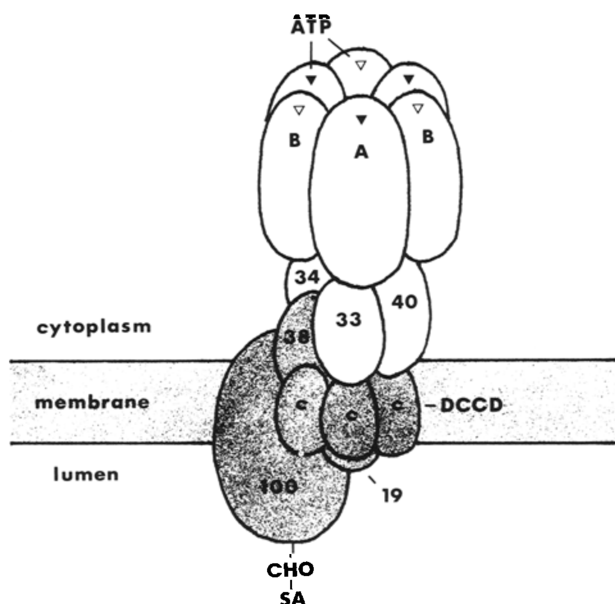

Fig. 1. Structural model of the coated vesicle (H^+)-ATPase. The coated vesicle (H^+)-ATPase is composed of two domains. The peripheral V_1 domain (in white) contains the A-, B-, 40-, 34-, and 33-kDa subunits in the stoichiometry $A_3B_340_134_133_1$ and is oriented toward the cytoplasmic side of the membrane (Arai *et al.*, 1988; Adachi *et al.*, 1990a,b). The A subunits possess the catalytic nucleotide binding sites (closed triangles) (Arai *et al.*, 1987b; Feng and Forgac, 1992), while the B subunits also appear to possess nucleotide binding sites (open triangles) (Adachi *et al.*, 1990a) of unknown function. The V_1 domain appears to be attached to the integral V_0 sector via the 40-, 34-, and 33-kDa "accessory" subunits (Adachi *et al.*, 1990b), with the 40-kDa subunit contributing to the stability of the complex but not essential for coupling of ATP hydrolysis and proton translocation (Puopolo *et al.*, 1992b). The integral V_0 domain (shaded) has the structure $100_138_119_1c_6$ (Arai *et al.*, 1988; Zhang and Forgac, 1992). The 17- and 19-kDa subunits are highly hydrophobic proteins but possess some luminal mass, while the 100-kDa subunit is a transmembrane glycoprotein with most of its hydrophilic mass on the luminal side of the membrane (Arai *et al.*, 1988; Adachi *et al.*, 1990b). The 38-kDa subunit, while tightly bound to V_0, is not labeled by the photactivated hydrophobic reagent [^{125}I]TID (Arai *et al.*, 1988) and does not possess any putative transmembrane helices (Wang *et al.*, 1988). It thus appears to be entirely anchored to the membrane through interactions with the other V_0 subunits. Although possessing all the information required for proton translocation, the isolated V_0 domain does not appear to be a functional proton channel (Zhang and Forgac, 1992). The 17-kDa c subunit is responsible for the DCCD-sensitivity of proton translocation (Arai *et al.*, 1987a), but may not be sufficient to form a proton channel.

nucleotide binding sites are located on the A subunits while the B subunits are also believed to contribute to ATP binding. The nucleotide binding domain is connected to the membrane via the 40-, 34-, and 33-kDa "accessory" subunits. The V_0 domain is responsible for proton translocation while the 17-kDa c subunit is responsible for the DCCD-sensitivity of proton transport. The 100-kDa subunit is a transmembrane glycoprotein of as yet unknown function. The vacuolar (H^+)-ATPases thus closely resemble the F-type (H^+)-ATPases of mitochondria, chloroplasts, and bacteria, with which they share sequence homology. Unlike the F-type ATPases, however, the V_1 and V_0 domains do not function independently. The possible significance of this finding to the mechanisms of regulating the activity of the vacuolar (H^+)-ATPases is discussed in Sec. 6.

3.2. Subunit Composition, Stoichiometry, and Domain Structure

The coated vesicle (H^+)-ATPase is composed of nine subunits of molecular mass 100, 73(A), 58(B), 40, 38, 34, 33, 19, and 17(c) kDa (Arai *et al.*, 1987b; Xie and Stone, 1986). We have demonstrated that this set of nine polypeptides is immunoprecipitated as a single macromolecular complex using monoclonal antibodies which recognize the native enzyme (Arai *et al.*, 1987b). Using quantiative amino acid analysis, we have shown that these subunits are present in a stoichiometry of $100_1A_3B_340_138_134_133_119_117_6$ (Arai *et al.*, 1988). A similar subunit composition has been observed for the vacuolar (H^+)-ATPases of kidney microsomes (Gluck and Caldwell, 1987), chromaffin granules (Moriyama and Nelson, 1989), *Neurospora* (Bowman *et al.*, 1989), yeast (Kane *et al.*, 1989), and plants (Parry *et al.*, 1989; Lai *et al.*, 1988).

The (H^+)-ATPase complex is divided into two structural domains. The peripheral V_1 domain, which has a molecular weight of 500,000, has the structure $A_3B_340_134_133_1$ (Arai *et al.*, 1988) and can be removed from the membrane with chaotropic agents such as KI and KNO_3 (Arai *et al.*, 1989; Adachi *et al.*, 1990b). This dissociation of the peripheral complex is activated by submicromolar concentrations of ATP (Arai *et al.*, 1989). Interestingly, dissociation of the peripheral subunits under these conditions results in their initial release from the membrane as monomers which on removal of the chaotropic agent and ATP are capable of reassembling into various subcomplexes (Puopolo and Forgac, 1990; Puopolo *et al.*, 1992b), as discussed in Sec. 3.5.

The integral V_0 domain contains four subunits in a stoichiometry of $100_138_119_1c_6$ (Arai *et al.*, 1988). These polypeptides remain assembled into a complex of molecular mass approximately 250 kDa following

removal of the peripheral subunits and solubilization of the membrane with $C_{12}E_9$ (Zhang *et al.*, 1992).

3.3. Topography and Glycosylation

Topographical studies have indicated that the entire V_1 domain is oriented towards the cytoplasmic side of the membrane (Arai *et al.*, 1988; Adachi *et al.*, 1990a). Thus, all of the V_1 subunits can be labeled in intact coated vesicles with membrane-impermeant reagents under conditions where only the cytoplasmic surface is exposed (Arai *et al.*, 1988). In addition, using a uniformly sided population of reconstituted vesicles, we have shown that all of the V_1 subunits can be cleaved by trypsin from the cytoplasmic side of the membrane (Adachi *et al.*, 1990a). Cleavage of the A and B subunits occurs at discrete sites 1–2 kDa from the amino terminus.

Of the V_0 subunits, at least the 100- and 38-kDa subunits are exposed on the cytoplasmic side of the membrane based on their labeling by impermeant reagents and their sensitivity to trypsin added from the cytoplasmic surface (Arai *et al.*, 1988; Adachi *et al.*, 1990a). Tryptic cleavage of these proteins again occurs at discrete sites to generate carboxyl terminal fragments of 80 and 36 kDa, respectively (Adachi *et al.*, 1990a). The 100-, 19-, and 17-kDa subunits also appear to possess domains exposed within the lumen of the coated vesicle since these subunits show a significant increase in labeling by impermeant reagents upon detergent permeabilization of intact coated vesicles (Arai *et al.*, 1988).

The 100-kDa subunit is glycosylated based upon its ability to bind peanut lectin (Adachi *et al.*, 1990b). That lectin binding is only observed following treatment with neuraminidase indicates that the carbohydrate chain terminates in sialic acid, which normally masks the lectin binding site. Since covalently bound siliac acid is found exclusively on the noncytoplasmic surface of the membrane, this result provides further evidence for a luminal domain of the 100-kDa subunit and indicates this polypeptide is a transmembrane glycoprotein (Adachi *et al.*, 1990b). Cloning and sequence analysis have confirmed this conclusion and have further indicated that this protein is divided into an amino terminal hydrophilic half and a carboxyl terminal hydrophobic portion made up of six putative transmembrane helices (Perin *et al.*, 1991).

3.4. Hydrophobic Domains

Both the c and the 19-kDa subunits are highly hydrophobic proteins which appear to be almost entirely burined in the bilayer. This is based upon their extraction by organic solvents (Arai *et al.*, 1987a), their high proportion of nonpolar amino acids (Arai *et al.*, 1988), and, in the case of the c subunit, on the sequence derived for the corresponding chromaffin granule protein (Mandel *et al.*, 1988). Heavy labeling of the c subunit with the photoactivated hydrophobic reagent [^{125}I]TID confirms that this protein is in extensive contact with the lipid bilayer (Arai *et al.*, 1988).

Interestingly, [^{125}I]TID shows no labeling of the 38- and 19-kDa subunits while a small amount of labeling of the 100-kDa subunit is observed (Arai *et al.*, 1988). While this is consistent with the transmembrane orientation of the 100-kDa polypeptide, it suggests that the 19-kDa subunit is shielded from contact with the lipid bilayer through interaction with other membrane spanning domains, most likely contributed by the c and 100-kDa subunits. The absence of [^{125}]TID labeling of the 38-kDa polypeptide is also consistent with the sequence derived from the corresponding chromaffin granule protein, which shows no putative transmembrane helices (Wang *et al.*, 1988). The 38-kDa subunit therefore appears to be a peripheral polypeptide which remains tightly bound through protein–protein contacts to the integral V_0 domain following removal of the V_1 subunits.

3.5. Subunit Contacts and Partial Complexes

Crosslinking studies of the coated vesicle (H^+)-ATPase using the reversible crosslinking reagent DTSSP have revealed extensive contact regions between the A and B subunits as well as contact between the c subunit and each of the V_1 accessory subunits of molecular mass 40, 34, and 33 kDa (Adachi *et al.*, 1990b). These results are consistent with the model shown in Fig. 1 where the A and B subunits form a hexameric arrangement (although other arrangements with extensive contact between the A and B subunits are clearly possible) and where the accessory V_1 subunits form a bridge between the peripheral V_1 complex and the integral V_0 domain.

As mentioned in Sec. 3.2, dissociation of the V_1 subunits from the membrane with KI and ATP results in their initial release as monomers. Removal of the chaotropic agents in the presence of membranes containing V_0 results in the reassembly of functional V_1V_0 complexes (Puopolo and Forgac, 1990). If KI and ATP are removed in the absence of V_0 and reassembly is monitored by sedimentation on glycerol density

gradients, the following results are obtained. A subcomplex of molecular mass approximately 500 kDa containing the A, B, 34-, and 33-kDa in the relative stoichiometry of $A_3B_334_133_1$ is formed which can be separated from the monomeric subunits by sedimentation (Puopolo *et al.*, 1992b). This subcomplex completely lacks the 40-dDa subunit, which appears in the "monomeric" fraction together with approximately 50% of the A, B, and 33-kDa subunits. Interestingly, the monomeric fraction is almost completely devoid of the 34-kDa subunit (Puopolo *et al.*, 1992b).

Evidence for interaction between the 40- and 33-kDa subunits derives from immunoprecipitation experiments using the monoclonal antibody 1C–11G (Puopolo *et al.*, 1992b). This monoclonal antibody immunoprecipitates both the 40- and 33-kDa subunits from the "monomeric" fraction, suggesting that these two subunits actually form a complex. That the antibody is actually directed against an epitope expressed on the native 33-kDa subunit is indicated by its ability to also immunoprecipitate the $V_1(-40\,kDa)$ subcomplex (Puopolo *et al.*, 1992b). Thus, during *in vitro* reassembly, the 33-kDa subunit assembles either with the 40-kDa polypeptide or with the remaining V_1 subunits to form the $V_1(-40\,kDa)$ subcomplex.

The V_0 subunits, unlike the V_1 subunits, remain together as a complex of molecular mass approximately 250 kDa following removal of V_1 with KI and ATP (Zhang *et al.*, 1992). The V_0 subunits appear to be present in the same stoichiometry in the free V_0 domain as in the intact V_1V_0 complex and to have many of the same properties. Thus the c subunit in the free V_0 domain is extracted by organic solvents and labeled by [^{14}C]DCCD (see Sec. 4.3) as it is in V_1V_0. In addition, a monoclonal antibody has been isolated which recognizes the 100-kDa subunit in both complexes. Both the 100- and 38-kDa subunits show the same tryptic cleavage pattern in the free V_0 complex as in the intact V_1V_0, although the sensitivity to proteolysis appears to have increased for both subunits following removal of the V_1 domain (Zhang *et al.*, 1992). Thus, the cytosolic domains of these two subunits become more accessible to the aqueous phase in the absence of V_1.

4. SUBUNIT AND DOMAIN FUNCTION AND IDENTIFICATION OF CATALYTIC RESIDUES

4.1. Nucleotide Binding Subunits

The A subunit has been shown to possess a nucleotide binding site required for catalysis based on its labeling by both [^{3}H]NEM and [^{14}C]NBD-Cl in an ATP protectable manner (Arai *et al.*, 1987b). Similar results have been obtained with vacuolar (H^+)-ATPases from a variety of other sources (Forgac, 1989). Further evidence supporting the role of both the A and B subunits in nucleotide binding has been provided by the protection of both subunits against tryptic cleavage in the presence of the nucleotide analog TNP-ATP (Adachi *et al.*, 1990a).

The strongest evidence indicating the role of the A and B subunits in nucleotide binding, however, has come from the cloning and sequence analysis of these proteins from a variety of sources. As first demonstrated by Zimniak *et al.* (1988), E. J. Bowman *et al.* (1988b), and B. J. Bowman *et al.*, (1988), the A and B subunits from plant and *Neurospora* vacuoles are evolutionarily related both to each other and to the alpha and beta subunits of the F-type (H^+)-ATPases. Extensive data from chemical modification sudies and mutational analysis have indicated that both the alpha and beta subunits of F_1 possess nucleotide binding sites, with the catalytic ATP binding sites being located on the beta subunit (Penefsky and Cross, 1991; Ysern *et al.*, 1988; Senior, 1988). The greatest sequence conservation between the vacuolar and F-type (H^+)-ATPase subunits in fact occurs in regions which appear to be critical for nucleotide binding (Zimniak *et al.*, 1988; Penefksy and Cross, 1991). Thus all four proteins involved in nucleotide binding by these two classes of (H^+)-ATPase appear to have been derived from a common evolutionary ancestor.

Like the vacuolar (H^+)-ATPases from other sources, the A and B subunits of the coated vesicle (H^+)-ATPase show similar sequence homology (Puopolo *et al.*, 1991; 1992a; Sudhof *et al.*, 1989). The A subunit from bovine appears to be encoded by a single gene which gives rise to a single transcript in all tissues tested (Puopolo *et al.*, 1991). The bovine B subunit, on the other hand, appears to be encoded by at least two, and possibly three, genes, which give rise to multiple transcripts in a tissue-specific manner (Puopolo *et al.*, 1992a). Thus, expression of both a 3.2- and 2.0-kb mRNA can be detected in all tissues examined except brain where expression of only the 3.2-kb message can be detected. Southern analysis is also consistent with the existence of multiple genes encoding the B subunit (Puopolo *et al.*, 1992a). The exact relation between the transcripts observed and the possible function of these transcripts in, for

example, encoding organelle-specific isoform remains to be determined.

4.2. Identification of Catalytically Important Residues in the A Subunit

We have recently identified the cysteine residue responsible for the sensitivity of the vacuolar (H^+)-ATPases to sulfhydryl reagents (Feng and Forgac, 1992). We demonstrated that, in addition to NEM, the coated vesicle (H^+)-ATPase was also inhibited by cystine in an ATP protectable manner. Unlike NEM, however, inhibition by cystine is reversible upon treatment with reducing agents, such as DTT. That cystine inhibits activity by formation of a disulfide bond with the same cysteine residue that reacts with NEM was demonstrated by the fact that extensive treatment of the cystine-reacted enzyme with NEM followed by treatment with DTT restores activity of the coated vesicle (H^+)-ATPase. We then took advantage of this protection of the NEM-reactive cysteine by cystine to selectively label this residue. The catalytic cysteine was first protected with cystine, the enzyme reacted extensively with NEM, the disulfide bond reduced with DTT, and the catalytic cysteine residue labeled with fluorescein maleimide. Under these conditions, Cys 254 fluorescein maleimide labels only the 73-kDa A subunit. When the labeled A subunit is eluted from the gel and subjected to cleavage by trypsin, a single 3.9-kDa fragment is labeled. We demonstrated that the cysteine residue labeled in this fragment corresponds to Cys 254 (Feng and Forgac, 1992), which is located in the Walker consensus "A" sequence GXGKTV (Walker *et al.*, 1985). This residue is in fact a cysteine in all of the vacuolar (H^+)-ATPase A subunit sequences obtained thus far (Puopolo *et al.*, 1991), whereas it is a valine residue in the F-type ATPases and a serine in the Archaebacterial (H^+)-ATPases (Penefsky and Cross, 1991), consistent with the insensitivity of the latter two classes to sulfhydryl reagents. As discussed in Sec. 2.2, we have demonstrated that this same catalytic cysteine residue is responsible for the sensitivity of the vacuolar (H^+)-ATPases to NBD-Cl.

4.3. DCCD-Reactive Proteolipid

The 17-kDa c subunit is responsible for the DCCD-sensitivity of the coated vesicle (H^+)-ATPase (Arai *et al.*, 1987a). Thus, reaction of the c subunit with DCCD results in complete loss of proton transport activity, although ATP hydrolysis is only inhibited if the (H^+)-ATPase is embedded in a lipid bilayer (either the native membrane or a reconstituted vesicle). The latter result suggests that in a detergent micelle, ATP hydrolysis and proton transport become uncoupled such that the proton conduction pathway can be blocked without effect on ATP cleavage. As discussed in Sec. 6, this is one of a series of treatments which results in a complete or partial uncoupling of proton transport and ATP hydrolysis.

Comparison of the stoichiometry of DCCD labeling with the degree of inhibition suggests that complete blockage of proton conduction can be achieved after reaction of only one-sixth of the c subunits with DCCD (Arai *et al.*, 1987a). As with the c subunit of F_0, this result suggests that formation of a passive proton pore requires the cooperative interaction of six copies of the c subunit, in agreement with the measured subunit stoichiometry (Arai *et al.*, 1988).

Cloning and sequence analysis of the c subunit of the chromaffin granule (H^+)-ATPase has confirmed that this polypeptide is the vacuolar counterpart of the F_0 c subunit which appears to have evolved from the latter by gene duplication (Mandel *et al.*, 1988). Interestingly, despite being twice the size of the 8-kDa F_0 protein, the vacuolar c subunit has only a single buried aspartate residue which is the likely site of reaction with DCCD. Thus, because there are most likely 10–12 copies of the c subunit in F_0 (Foster and Fillingame, 1982), the number of putative transmembrane helices contributed by the c subunit has been conserved, but the number of buried carboxyl groups has been reduced by a factor of 2. This has led to a very interesting speculation concerning the mechanism by which the H^+/ATP stoichiometry of the V and F-type (H^+)-ATPases was altered during the course of their evolution (Cross and Taiz, 1990).

One report has suggested that the c subunit alone is sufficient to form a proton channel (Sun *et al.*, 1987). Based on the homology between the vacuolar and F_0 c subunits (Mandel *et al.*, 1988), and on the extensive genetic and biochemical evidence indicating that the c subunit of F_0 is not sufficient to form a proton pore (Cain and Simoni, 1986; Schneider and Altendorf, 1987), it seems most likely that additional subunits will also be required for proton conduction through the V_0 domain. One possible candidate for the vacuolar counterpart to the a subunit, which has been shown to be critical for proton conduction through F_0, is the 19-kDa polypeptide. Like the a subunit, this

protein is present in one copy per complex and, based on its amino acid composition, is extensively buried in the bilayer (Arai *et al.*, 1988). The role of the 19-kDa subunit in proton conduction through V_0 is currently under study in our laboratory.

4.4. Function of Accessory Subunits

To address the role of the accessory subunits of the coated vesicle (H^+)-ATPase, we have developed a protocol which allows us to reassemble a (H^+)-ATPase from the dissociated V_1 subunits and the intact V_0 domain which is functional in both ATP hydrolysis and proton translocation (Puopolo and Forgac, 1990). Reassembly involves attachment of the complete complement of V_1 subunits to the V_0 sector, is time- and protein concentration-dependent, and gives rise to a reassembled (H^+)-ATPase with inhibitor sensitivities identical to the native enzyme.

Recently, we have employed this reassembly system to address the role of the 40- and 34-kDa subunits (Puopolo *et al.*, 1992b). As explained in Sec. 3.5, we have isolated a $V_1(-40\,\text{kDa})$ subcomplex which contains the A-, B-, 34-, and 33-kDa subunits. This subcomplex, although devoid of ATPase activity, is able to reassemble onto the V_0 domain to give a complex possessing approximately 50% of the proton transport activity obtained using the complete complement of V_1 and V_0 subunits (Puopolo *et al.*, 1992b). Reassembly requires that the $V_1(-40\,\text{kDa})$ subcomplex first be dissociated with KI and MgATP followed by reassembly in the presence of V_0. Thus the assembled subcomplex appears unable to bind to the membrane sector. Similarly, addition of the isolated 40-kDa subunit to the reassembly mixture is without effect on activity unless the $V_1(-40\,\text{kDa})$ subcomplex is first dissociated, under which conditions addition of the 40-kDa subunit restores activity to maximal levels. The binding sites for both V_0 and for the 40-kDa subunit thus appear to be inaccessible in the assembled V_1(-40 kDa) subcomplex.

The monomeric V_1 fraction lacking the 34-kDa subunit obtained by sedimentation (see Sec. 3.5) is also competent to reassemble with V_0 to give a partially active complex (Puopolo *et al.*, 1992b). The absence of either the 40- or 34-kDa subunits, however, makes the reassembled complexes unstable to detergent solubilization and immunoprecipitation. The results thus suggest that although not absolutely required for coupling of ATP hydrolysis and proton transport, both the 40- and 34-kDa subunits are required for stability and maximal activity of the coated vesicle (H^+)-ATPase. It is thus possible that these subunits play some role in regulation of either assembly or activity of the vacuolar (H^+)-ATPases *in vivo*.

4.5. Functional Properties of the V_1 and V_0 Domains

Unlike the F-type (H^+)-ATPases, the V_1 and V_0 domains of the coated vesicle (H^+)-ATPase do not appear to function independently. Thus the $V_1(-40\,\text{kDa})$ subcomplex is inactive with respect to ATPase activity (Puopolo *et al.*, 1992b), and no conditions have yet been identified that allow binding of the 40-kDa subunit to this subcomplex. A low level of Ca^{2+}-activated ATPase activity has been reported for some combinations of the peripheral subunits (Xie and Stone, 1986), but the fact that these complexes are unable to hydrolyze MgATP and that Ca^{2+} is unable to support proton transport by the coated vesicle (H^+)-ATPase makes the significance of these findings uncertain.

Similarly, the V_0 domain, unlike F_0, does not appear to be capable of carrying our passive, DCCD-inhibitable proton conduction (Zhang *et al.*, 1992). Proton conductance was measured in both native membranes from which the V_1 subunits had been dissociated and in reconstituted vesicles containing the isolated V_0 domain by uptake of the fluorescent dye ACMA in response to a K^+- and valinomycin-induced membrane potential. In neither case was any DCCD-inhibitable proton conduction detected, despite the fact that proton conduction could readily be detected on addition of the proton ionophore CCCP and that the V_0 domain was still competent to reassemble with the V_1 subunits to give a functional (H^+)-ATPase. These results suggest that one or more of the V_0 subunits may be suppressing proton conduction through V_0 (Zhang and Forgac, 1992). The possible role of such suppression *in vivo* is discussed in Sec. 6.

5. ASSEMBLY OF THE COATED VESICLE (H^+)-ATPASE

Using immunoprecipitation by antibodies raised against the bovine coated vesicle (H^+)-ATPase, we have investigated the biosynthesis and assembly of the vacuolar (H^+)-ATPases in a bovine kidney cell line, MDBK cells (M. Sczekan and M. Forgac, manuscript in preparation). We have found that fully assembled

complexes can be detected as early as 10 min after the initiation of labeling, and that assembly is not blocked by incubation at 15°C or treatment with brefeldin A under conditions where transport between the endoplasmic reticulum and the Golgi is disrupted. These results suggest that assembly of the V_1 and V_0 domains can occur in the ER. In addition, a soluble pool of assembled V_1 complexes can be detected which do not appear to be an obligate precursor to the complexes assembled in the ER. Thus a pool of assembled V_1 complexes which are not attached to the V_0 domain appears to exist at steady state. The possible role of such a soluble pool of V_1 complexes in regulating acidification is discussed below. Assembly of the vacuolar (H^+)-ATPase in yeast has recently been described by Kane *et al.*, (1992) and is discussed on p. 383 of this volume.

6. REGULATION OF COATED VESICLE ACIDIFICATION

While it is clear that cells are able to differentially modulate the pH of the various intracellular compartments (Forgac, 1989), the mechanisms by which this is accomplished remain uncertain. As discussed in Sec. 1.3, endocytic coated vesicles appear not to undergo acidification, but this is not due to the complete absence of vacuolar proton pumps. Thus, immunocytochemical analysis indicates that at least the A subunit is present bound to this compartment (Marquez-Sterling *et al.*, 1991). It is possible, however, that one or more of the (H^+)-ATPase subunits may be missing from the (H^+)-ATPases present in endocytic coated vesicles and that modulation occurs by addition of the missing subunit upon entry of the (H^+)-ATPase into CURL. One possible candidate for such a regulatory subunit is the 100-kDa polypeptide, which is present in the coated vesicle (H^+)-ATPase and other vacuolar (H^+)-ATPases but appears to be absent from the V-ATPase present in the plasma membrane of kidney cells (Gluck and Caldwell, 1987). Alternatively, regulation may be accomplished through substitution of organelle-specific isoforms of particular (H^+)-ATPase subunits. The existence of multiple isoforms of the B subunit in human (Bernasconi *et al.*, 1990) and bovine (Puopolo *et al.*, 1992a) has recently been demonstrated.

The degree of vacuolar acidification may in some cases be controlled by changes in the number of vacuolar (H^+)-ATPases in a particular compartment. This mechanism appears to operate in regulating acid secretion across the lumenal membrane of the intercalated cells of the mammalian kidney (Gluck *et al.*, 1982). In this case, the density of pumps in the lumenal membrane is controlled by reversible fusion with the plasma membrane of intracellular vesicles containing a high density of vacuolar proton pumps.

A third possible mechanism for regulating vacuolar acidification involves control of coupling between ATP hydrolysis and proton translocation. As discussed above, there are several experimental conditions which have been shown to alter the tightness of this coupling for the coated vesicle (H^+)-ATPase, including high concentrations of ATP (Arai *et al.*, 1989), partial proteolysis (Adachi *et al.*, 1990a), and detergent solubilization (Arai *et al.*, 1987a). These results suggest that the enzyme may be poised in a state where the stoichiometry of proton transport can readily be altered in response to the appropriate intracellular signal. The nature of such an intracellular signal is presently unknown.

A fourth possible regulatory mechanism is suggested by the fact that the separated V_1 and V_0 domains appear to be inactive with respect to ATP hydrolysis and proton conduction (Puopolo *et al.*, 1992b; Zhang *et al.*, 1992) (see Sec. 4.5). This mechanism would involve control of ATP-driven proton transport by controlling attachment of the V_1 and V_0 domains. Such a mechanism would likely necessitate that at least the V_0 domain was not functional as a passive proton channel, since otherwise in any compartment containing multiple copies of V_0, no significant pH gradient could be achieved until all of the V_0 sites had been occupied. Further evidence consistent with this model comes from the observation that coated vesicles appear to contain an excess of V_0 domains over those required to form functional V_1V_0 complexes (Zhang and Forgac, 1992) and from the fact that a soluble pool of V_1 domains exist in MDBK cells (M. Sczekan and M. Forgac, manuscript in preparation).

A final mechanism of controlling vacuolar pH involves control of the chloride channel required for acidification to occur. In fact, we have recently demonstrated that both chloride conductance and ATP-dependent acidification in coated vesicles are modulated by a protein kinase A-dependent phosphorylation and that this effect is the result of alteration in the activity of the chloride channel (Mulberg *et al.*, 1991). Which of these mechanisms actually plays an important role in regulating vacuolar acidification *in vivo* remains to be determined.

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