Identification and Reconstitution of an Isoform of the 116-kDa Subunit of the Vacuolar Proton Translocating ATPase*

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We have identified a cDNA encoding an isoform of the 116-kDa subunit of the bovine vacuolar proton translocating ATPase. The predicted protein sequence of the new isoform, designated a2, consists of 854 amino acids with a calculated molecular mass of 98,010 Da; it has approximately 50% identity to the original isoform (a1) we described (Peng, S.-B., Crider, B. P., Xie, X.-S., and Stone, D.K. (1994) J. Biol. Chem. 269, 17262–17266). Sequence comparison indicates that the a2 isoform is the bovine homologue of a 116-kDa polypeptide identified in mouse as an immune regulatory factor (Lee, C.-K., Ghoshal, K., and Beaman, K.D. (1990) Mol. Immunol. 27, 1137–1144). The bovine a1 and a2 isoforms share strikingly similar structures with hydrophilic amino-terminal halves that are composed of more than 30% charged residues and hydrophobic carboxyl-terminal halves that contain 6–8 transmembrane regions. Northern blot analysis demonstrates that isoform a2 is highly expressed in lung, kidney, and spleen. To determine the possible role of the a2 isoform in vacuolar proton pump function, we purified from bovine lung a vacuolar pump proton channel (VO) containing isoform a2. This VO conducts bafilomycin-sensitive proton flow after reconstitution and acid activation, and supports proton pumping activity after assembly with the catalytic sector (V1) of vacuolar-type proton translocating ATPase (V-ATPase) and sub-58-kDa doublet, a 50–57-kDa polypeptide heterodimer required for V-ATPase function. These data indicate that the a2 isoform of the 116-kDa polypeptide functions as part of the proton channel of V-ATPases.

Vacular-type proton translocating ATPases (V-ATPases) are widely distributed in eukaryotic cells where they are found in most organelles. In addition, these proton pumps are localized to plasma membranes of epithelia, macrophages, and specialized polarized cells. V-ATPases have been shown to control osteoclast-mediated bone reabsorption and renal acidification and are thereby involved in the pathogenesis of osteoporosis and the systemic acidosis of uremia (1–3). Key to understanding the regulation of these diversely distributed proton pumps is a basic investigation of the structure of the enzymes and delineation of the roles of the individual subunits in pump function. The primary structures of V pump subunits, as well as the overall subunit composition and quartenary structures of the holoenzyme, are highly conserved through species as evolutionarily diverse as Archaeabacteria, Caenorhabditis elegans, and Homo sapiens. In its simplest form, the V pump of Enterococcus hirae is composed of 11 subunits, some of which are homologues of the subunits of the V-type proton pump of clathrin-coated vesicles of bovine brain (4).

Structurally, V-ATPases resemble F1 Fo-type ATP synthases in that they are complex hetero-oligomers with two functional domains: an ATP-hydrolytic sector (V1 or Vc) that is peripheral to the membrane, and a transmembranous proton channel (V0 or Vp). The V1 domain of the V pump of clathrin-coated vesicles consists of essential, core subunits of 70, 58, 40, 34, 33, 14, and 10 kDa, designated A, B, C, D, E, G, and F, respectively. In addition, a key regulatory element, the sub-58-kDa doublet (SFD), consists of polypeptides of 57 and 50 kDa, activates V1 and functionally couples ATP hydrolysis to proton flow through the transmembranous sector, VO (5, 6). Separation of V1 from VO results in marked changes in the functions of these two domains. Although native holoenzyme hydrolyzes MgATP at a rate 3-fold higher than CaATP, isolated V1 hydrolyzes ATP only in the presence of Ca2+; Mg2+, in fact, inhibits ATP hydrolysis catalyzed by V1 (7). In addition, the proton channel, VO, is closed after separation from V1, and requires incubation at an acidic pH to restore proton flow, which is inhibitable by bafilomycin A1, a V-ATPase specific inhibitor (8).

Although the subunit composition of V1 is now well defined, there are conflicting reports regarding the components of VO. All investigators find a 17-kDa polypeptide (subunit c), as well as a 39-kDa subunit in VO preparations. In addition, the VO component of the proton pump of clathrin-coated vesicles contains a 116-kDa polypeptide, and a polypeptide of this mass, or a smaller homologue, has been demonstrated in most V-pump preparations. The function of the 116-kDa subunit is not defined, but its predicted structure consists of 6–8 transmembranous sectors, suggesting that it may function similar to subunit a of Fp.

Additional structural complexity exists in V-ATPases in the form of subunit isoforms. Two forms of subunit A (9–11) and subunit B (12, 13) have been identified. Subunit G, a recently identified subunit that is required for ATP hydrolysis, has two isoforms that differ in tissue distribution and function (14). Most recently, we have demonstrated that the 50- and 57-kDa isoforms of the 116-kDa subunit are a contributor to VO.
polypeptides of SFD are isoforms resulting through alternative mRNA splicing (7). In addition, three forms of the c subunit of V$_{10}$ have been shown to be required for V-pump function in yeast (15).

The 116-kDa subunit of V$_{10}$, the subject of this investigation, appears to have the greatest degree of isoform diversity of all V-pump components. This diversity arises through two mechanisms. First, alternative splicing of mRNA results in two forms of the subunit prevalent in brain (a$_1$ isoform). This alternative splicing results in changes within a predicted protease sensitivity motif (PEST site, a region enriched in proline, glutamic acid, serine, or threonine residues), implying differences in the biological half lives of the two isoforms (16, 17). Second, higher organisms have separate genes that encode distinct isoforms of the 116-kDa subunit. In yeast, two such genes, designated VPH1 (20) and STV1 (21), encode proteins with amino acid sequence homology to the mammalian 116-kDa polypeptide. Cumulative evidence suggests that three separate genes encode forms of the 116-kDa subunit in mammalian species. In addition to the a$_1$ isoform of bovine brain, a related homologue has been identified in murine T cells, and a third form in human osteoclasts. Although interspecies comparison of primary sequence complicates this point, it is notable that the sequence divergence between these bovine, murine, and human forms of the 116-kDa subunit greatly exceeds the divergence observed in all other pump subunits. Moreover, recent experiments have demonstrated three distinct genes encoding 116-kDa isoforms in chicken.  

Of these putative forms of the 116-kDa subunit, the isoform isolated from murine T cells has not been identified as a V-pump component. In fact, the cDNA encoding this subunit was isolated by a strategy designed to identify novel immune regulatory factors. To investigate the function of this isoform and to determine its relationship to V-pump function, we have cloned and sequenced the cDNA encoding this isoform of the 116-kDa subunit. It shares only 50% identity to the 116-kDa subunit of bovine brain that we described previously, but has 91.6% identity to the 116-kDa isoform of murine T cells. The two polypeptides have strikingly similar structure, with hydrophilic amino-terminal halves that are composed of $>$30% charged residues and hydrophobic carboxyl-terminal halves that contain 6–8 transmembrane regions. The new isoform, designated a$_2$, copurifies with vacuolar proton channel from lung. Reconstitution experiments demonstrate that it is associated with functional V$_{10}$, indicating that it is a genuine isoform of the 116-kDa subunit of V-pumps.  

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 DNA ligase, and a nick translation kit for DNA probe labeling were purchased from Boehringer Mannheim; the GeneAmp polymerase chain reaction (PCR) reagent kit with DNA polymerase and DNA sequencing mate-rial and reagents were from Perkin-Elmer; a TA cloning kit containing vector, pCR 2.1, and DNA ligase were from Invitrogen; Escherichia coli strains XLI-Blue-MRF$^+$ and XLOLL and helper phage R408 were from Stratagene; radioactive materials and an ECL kit for Western blot analysis were from Amersham Pharmacia Biotech; nitrocellulose membranes for plaque lift were from Millipore Corp., and chemicals for SDS-PAGE were from Bio-Rad. A bovine brain cDNA library in AZAP was the kind gift of Dr. Richard A. F. Dixon (The University of Texas Health Science Center at Houston). All other reagents were from Sigma.

**Synthesis of a 0.3-kb DNA Fragment by PCR**—Two oligonucleotide primers, 5'-TCIGG/CAA/AAACATGACG/C/GAA-3' and 5'-AATTG/C/T/CTTATGGCIGA/G/GTGTGTTG-3', were designed and synthesized in accord with two regions of protein sequences of 116-kDa subunits of vacuolar ATPases that are highly conserved in all species (16–21). Deoxyinosine (I) was used in the third position of some codons with a degeneracy of two or more. AZAP phage DNA from amplified bovine brain cDNA library was purified by standard procedure (22) and used as a template for PCR; this was performed with 20 ng/ml of each primer and 1 µg of purified AZAP DNA. PCR products of 0.3 kb were cloned into pCR 2.1 vector using a TA cloning kit from Invitrogen. The positive colonies from TA cloning were analyzed by DNA sequencing, and a 0.3-kb insert was excised with EcoRI digestion, purified by preparative agarose gel electrophoresis, and used to screen a bovine brain cDNA library.

**Cloning of Bovine Brain cDNA Library**—A bovine brain cDNA library in AZAP (insert size, $>$2.0 kb), transfected into E. coli, XLI-Blue-MRF$^+$, was screened with the 0.3-kb PCR product that had been labeled with $[^{32}P]$dCTP by nick translation. Screening of 2 $\times$ 10$^5$ individual phages was performed using a double-lift procedure wherein plaques were picked into the nitrocellulose membranes for 5 min in each lift. The membranes were prehybridized for at least 4 h at 60 °C in a solution containing 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.1 µg/ml of sheared salmon sperm DNA, and 0.1% SDS. Hybridization was performed at 50 °C overnight with the same solution plus labeled probe, which was added at a concentration of 5–10 $\times$ 10$^5$ cpm/ml of hybridization solution. The membranes were then washed for 15 min at room temperature first with 2 $\times$ SSC and 0.1% SDS, then with 0.5 $\times$ SSC and 0.1% SDS, and finally with 0.1 $\times$ SSC and 0.1% SDS. Autoradiography was performed with an intensifying screen at ~80 °C for 24–48 h. Duplicate positive clones were cored and rescreened through one or more cycles until purified colonies were obtained.

**Subcloning and DNA Sequencing**—Inserts of all positive clones were excised and cloned into pBluescript with the helper phage R408, as described (10). Plasmid DNA was prepared by alkaline lysis, and DNA sequencing reactions were performed using a Model 377 ABI Prism DNA sequencer and the manufacturer's reagents. All positive clones were sequenced in both orientations using M13 reverse, M13 (−21), and sequence-specific oligonucleotides as primers. DNA and protein data base analysis was performed using PC/Gene-based programs.

**Northern Blot Analysis**—Poly(A)$^+$ RNA (2 µg/lane) from different bovine tissues was denatured and fractionated by 1% formaldehyde-agarose gel electrophoresis, and transferred to a Zeta-probe blotting membrane (Bio-Rad). After baking at 80 °C in a vacuum oven for 1 h, the membrane was prehybridized for 4 h at 50 °C in a solution consisting of 50% formamide, 1.5 $\times$ saline/sodium phosphate/EDTA, 1% SDS, 0.5% nonfat dry milk, and 0.5 µg/ml of denatured salmon sperm DNA. A 0.6-kb cDNA fragment encoding the NH$_2$-terminal portion of isoform a$_2$ was labeled with $[^{32}P]$dCTP by nick translation and added to the hybridization solution at a concentration of 1 $\times$ 10$^5$ cpm/ml of solution. Hybridization was then carried out at 50 °C overnight. The membrane was sequentially washed for 15 min at room temperature with 2$\times$ SSC and 0.1% SDS, 0.5$\times$ SSC and 0.1% SDS, and 0.1$\times$ SSC and 0.1% SDS, respectively. A final wash was carried out at 60 °C for 30 min with 0.1$\times$ SSC plus 0.1% SDS, and autoradiography was performed with an intensifying screen at ~80 °C for 3–5 days.

**Antibody Preparation and Western Blot Analysis**—Isotype a$_2$-specific (CVRIRQRYYRKHLGT) and a$_2$-specific (CGTIPSFTMNTIPTKET) peptides were synthesized based upon the deduced protein sequences, coupled to keyhole limpet hemocyanin, and utilized for immunization of a New Zealand White rabbits to generate polyclonal antibodies, as described (23). The preparations of antibodies directed against the 70-kDa (subunit A) and 39-kDa subunits have been previously reported.

For Western blot analysis, protein samples were separated by 11% SDS-PAGE and transferred electrophoretically to nitrocellulose filters. Immunodetection was performed using immune serum at a 1:5000 dilution and an Amersham Pharmacia Biotech ECL Western blotting system.

**Isolation of Vacuolar Proton Channel (V$_{10}$) from Bovine Lung**—Microsomes were prepared from bovine lung by using the buffer solution and initial steps used to prepare clathrin-coated vesicles from bovine brain. Briefly, bovine lung (1 kg) was homogenized in a Waring blender in 2 liters of Buffer A, consisting of 100 mM MES (pH 6.5), 3 mM azide, 1 mM EGTA, and 0.5 mM MgCl$_2$. The homogenate was centrifuged at 180,000 $\times$ g for 1 h. The final crude, microsomal pellet was used for isolation of vacuolar proton pump and V$_{10}$, as follows. Membrane pellet (5 ml) was resuspended in 20 ml of Buffer A with 1% C$_2$E$_6$(polyoxyethylene 9-lauryl ether) and incubated on ice for 30 min. After centrifugation at 180,000 $\times$ g for 1 h, the resulting pellet was resuspended in Buffer A containing 1% sodium cholate. Centrifugation was repeated, and the final pellet was resuspended in 3 ml of 1% Zwittergent 3–16.
and incubated at room temperature for 1 h. After centrifugation at 180,000 g for 1 h, the supernatant (3 ml) was loaded on two 13-ml linear (15–30%) glycerol gradients prepared in Buffer G, consisting of 20 mM Tris-HCl (pH 7.5), 0.05% C12E9, 5 mM dithiothreitol, and 0.5 mM EDTA. After centrifugation at 180,000 g for 22 h at 4°C, fractions of 1 ml were harvested from the bottom of the tube, subjected to SDS-PAGE, Western blot, and proton pumping and/or proton channel activity analysis. For further purification, the peak fractions were combined, concentrated with a Millipore Ultrafree-MC centrifugal filter unit, and separated by a second 15–30% glycerol gradient centrifugation that was performed as described above.

Purification of Vpump, VO, and SFD from Bovine Brain—Clathrin-coated vesicles were prepared from batches of 30 bovine brains, and Vpump was solubilized with 1% C12E9 and purified to a specific activity of 14–16 µmol of P1 × mg protein−1 × min−1, as described (24). Vp, VO, and SFD were prepared as reported (7, 5).

Reconstitution—Reconstitutions of vacuolar proton pump and proton channel (VC) were performed by the freeze-thaw, cholate-dilution method using liposomes prepared from pure lipids. Stock solution of liposomes composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and cholesterol at a mass ratio of 4:2:2:2.76: 0:2.2:7 were prepared as described (25). Typically, 100 µl (0.5–0.8 mg of protein/ml) of purified proton pump or VC was mixed with 3 mg of liposomes and 26 µl of reconstitution buffer consisting of 6% sodium cholate, 0.05% C12E9, 0.5 M KCl, 0.5 M NaCl, 600 mM Na2HPO4, and 600 mM MgCl2. The mixture was frozen in liquid N2 for 2 min and then incubated at room temperature for 1 h. Proteoliposomes were diluted with 6 ml of 150 mM KCl, 20 mM Tricine (pH 7.5), 3 mM MgCl2, and 0.5 mM EDTA (dilution buffer); concentrated by centrifugation at 110,000 g for 1 h at 15 °C; and then resuspended with 50 µl of dilution buffer for proton transport assays described below. Acid activation of the latent proton conductance of reconstituted VC was performed as described (5), by incubation of 5 µl of proteoliposomes containing VC with 2 µl of 0.5 M MES (pH 5.0) for 1 h on ice.

Acridine Orange Absorbance Quenching—Proteoliposomes acidification was assessed by the measurement of acridine orange quenching in an SLM-Amino DW2-C dual wavelength spectrophotometer as described (25). Several positive clones, designated B2–2 lacked coding region for 9 amino acids at the 5′-end, and the other clones had inserts of 1.5–2.5 kb. All of the clones had identical sequences in overlapping regions. The full sequence of clone B31–1 includes a 2565-base pair open reading frame and untranslated regions of 174 and 1703 base pairs at the 5′- and 3′-ends, respectively.

Analysis of the Deduced Amino Acid Sequence—Translation of the open reading frame of clone B31–1 predicts an 854-amino acid polypeptide with calculated molecular mass of 98,010 Da, which is close to the mass of 96,301 Da of isoform a1. Three potential N-glycosylation sites are present at residues 43, 157, and 505. The calculated isoelectric point is 5.89. This isoform shares 50% identity with the a1 isoform that we described previously (16, 17). Kyte-Doolittle (27) analysis reveals that the two isoforms have strikingly similar structures, with two characteristic domains: a hydrophilic amino-terminal half that is composed of more than 30% charged residues, and a highly conserved and hydrophobic carboxyl-terminal half that contains 6–8 transmembrane regions. Data base searches demonstrated that isoform a2 shares 91.6% identity at the amino acid level with mouse J6B7, a putative immune regulatory protein from T cells (19) (Fig. 1). As shown, particularly high levels of conservation were observed in predicted transmembranous sectors.

Expression of mRNA for Isoform a2 in Different Tissues—The tissue distribution of mRNA encoding the a2 isoform was investigated by Northern blot analysis. Although two transcripts of approximate sizes of 3.4 and 5.4 kb were detected in all tissues (brain, heart, kidney, lung, and spleen), the absolute and relative abundances differed between tissues. High levels of expression were found in the kidney, lung and spleen, whereas the brain had very low levels of a2 transcripts (Fig. 2).

Identification of Isoform a2 as a Component of Vacuolar Proton Pump—To determine the relationship of the cloned cDNA to the 116-kDa component of vacuolar proton pump, we generated isoform a1−, and a2-specific, anti-peptide antibodies based on predicted amino acid sequence. As shown in Fig. 3, lane 3, the a2-specific antibody reacts with a minor portion of the 116-kDa band of highly purified bovine brain vacuolar proton pump, indicating the presence of isoform a2 in V-ATPase complex. The same enzyme reacts heavily with isoform a1, specific, anti-peptide antibody (Fig. 3, lane 2). This suggests that isoform a1 is the major form and a2 is the minor form of the vacuolar proton pump in the brain, which is in good accord with the results of Northern blot analysis we obtained in this study (Fig. 2) and in previous investigations (17).

Isoform a2 Is the Major Form in Lung and Co-purifies with VO—Whereas these findings are highly suggestive that the a2 isoform is present in subpopulation V-type proton pumps in bovine brain, the co-purification of the a1 and a2 isoforms precluded any direct investigation of whether the a2 isoform was associated with a functional proton pump. We therefore sought to find an alternative tissue source highly enriched in the a2 isoform. As demonstrated by Northern blot analysis in Fig. 2, mRNA for isoform a2 is present in high copy number in lung. We therefore attempted to isolate proton pump from bovine lung using the solubilization and purification procedure we developed for the V-type proton pump of clathrin-coated vesicles of bovine brain. Microsomes were prepared from freshly harvested bovine lung by homogenization and a differential centrifugation. For comparative purposes, freshly harvested bovine brain was processed identically. However, testing of V-pump activities in the two microsomal preparations revealed that the vesicles from lung had only 1% the specific activity of the vesicles from bovine brains, as assessed by ATP generated acridine orange quenching. To determine whether this relatively low activity was due to an intrinsic proteolysis of the pump from lung, we tested several different solutions for...
microsomal preparation. These included variances in pH from 6.5 to 7.5 and inclusion of proteinase inhibitors. The same results, however, were obtained. Moreover, C12E9, the detergent routinely utilized for the solubilization of the V pump of clathrin-coated vesicles, was ineffective in solubilizing bafilomycin-sensitive ATPase activity from lung microsomes. Numerous detergents were tested and we ultimately determined that the V-ATPase of lung was optimally solubilized with Zwittergent 3–16. Subsequently, purification of intact V pump was attempted by our standard protocol, which includes hydroxylapatite chromatography, and glycerol gradient centrifugation. Repeated attempts at purification, however, resulted in minuscule amounts of pump that migrated to the usual position in glycerol gradient centrifugation (data not shown). Instead, Western blot analysis using an anti-α2 isoform antibody revealed that the α2 isoform was present at roughly the midpoint of the 15–30% glycerol gradients, where isolated VO is typically found. As shown in Fig. 4, Western blot analysis demonstrated that isoform α2 and the 39-kDa-subunit (28), an identified component of VO, were located in the same fractions. These

FIG. 1. Alignment of isoforms of the 116-kDa subunit and homologous sequences from mammalian cells. The predicted amino acid sequence of bovine α2 (VBA2-BOVIN) is compared with those of mouse J6B7 (a putative immune regulatory factor), bovine (VBA1-BOVIN) and rat (VBA1-RAT) isoform α1, and putative isoform from human osteoclastoma (OC116HUMAN). Identical amino acids are designated by asterisks, and similar amino acid residues (defined by PC/GENE-based algorithms) are denoted by a dot. Predicted transmembranous sectors (27) for isoform α2 are underlined.

FIG. 2. Northern blot analysis. Poly(A+) RNA (2 μg) from bovine tissues was denatured and fractionated by 1% formaldehyde-agarose gel electrophoresis and hybridized with a 32P-labeled cDNA probe, as described under “Experimental Procedures.” Lanes 1–5 represent poly(A+) RNA from brain, heart, kidney, lung, and spleen, respectively.
fractions containing the 116-kDa subunit reacted weakly with the isoform a2-specific antibody (Fig. 4), indicating that isoform a2 is the major form of 116-kDa subunit in the bovine lung. In addition, none of the glycerol gradient fractions reacted with an antibody against the 70-kDa subunit, a component of V1 of vacuolar proton ATPases. The peak fractions were combined, concentrated with a Millipore Ultrafree-MC centrifugal filter unit, and further purified by a second glycerol gradient centrifugation step. After centrifugation, relatively pure (and active) VO was obtained, as shown in Fig. 5, lane 2. The purified VO contained at least three polypeptides with molecular masses of 116, 39, and 17 kDa. Also observed was a polypeptide with a molecular mass of about 140 kDa. Whether this is a genuine VO component or a contaminant will require additional study. To date, we have been unable to achieve a higher degree of purification.

**V-ATPase Isoform a2 Conducts Proton Flow**—In order to determine whether the purified a2-containing VO could function as a proton channel, we performed reconstitution and acid activation experiments. As reported previously, VO isolated from bovine brain is closed to proton flow, but a latent proton conductance can be activated by briefly exposing the channel to an acidic pH. As shown in Fig. 6, isolated and reconstituted VO of bovine lung behaves similarly. Isolated VO does not conduct protons (trace 2), but acid pretreatment of VO activates a latent proton conductance (trace 4), which is inhibited by bafilomycin (trace 3). This fraction cannot support proton pumping when ATP is present in the reaction (trace 1), which further indicates that the isolated fraction does not contain functional V1.

**Reconstitution of Proton Pumping Activity with VO, V1, and SFD**—To determine whether VO prepared from bovine lung could function in ATP driven proton pumping, we reassembled VO from bovine lung, with V1 and SFD from bovine brain (5). As shown in Fig. 7, neither V1 plus SFD (trace 1), nor VO plus SFD (trace 2), could support ATP-dependent proton pumping as assessed by ATP generated acridine orange quenching. However, reassembly of V1, VO, and SFD results in a complex capable of supporting significant ATP-dependent proton pumping (trace 4). The reconstituted proton pumping activity was inhibited by 3 nM bafilomycin (trace 3).

**DISCUSSION**

V-ATPases are distributed among most intracellular organelles of both constitutive and regulated secretory pathways. It is thus to be expected that these pumps are highly regulated. In this regard, a steep intraorganelle pH gradient exists in the constitutive pathway, with lysosomes having a pH of 4.5; endosomes, pH 5.2; and terminal stack of Golgi membranes, a pH of almost 7.0 (29, 30). Whereas the pH of these compartments is probably statically maintained at these set points, the pH within organelles of the regulated secretory pathway is under a more dynamic regulation. Thus, the pH of early mast cell granules is about 5.5, whereas the pH of these organelles rises to 7.0 after processing of vesicle contents (31). The basis for these differences in pump function is not well established, but may owe to molecular diversity of V-ATPases.

**FIG. 3.** SDS-PAGE and Western blot analysis of the highly purified vacuolar proton ATPase from bovine brain. Lane 1, enzyme (3 μg) was subjected to 8% SDS-PAGE followed Coomassie Blue staining; lanes 2 and 3, Western blot analysis utilizing a1- and a2-specific anti-peptide antibodies, respectively.

**FIG. 4.** Western blot analysis of partial purified proton channel (VO) from bovine lung. The fractions from glycerol gradient centrifugation were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes for analysis. Lanes 1–12 are fractions from the bottom (lane 1) through the top (lane 12) of the gradient; P is purified vacuolar ATPase from bovine brain. Panels A–D illustrate Western blots performed with different antibodies: A, a2-specific antibody; B, a1-specific antibody; C, antibody against recombinant 39-kDa subunits; D, anti-70-kDa antibody (10).

**FIG. 5.** SDS-PAGE of purified, active VO, from lung and V-ATPase holoenzyme from brain. SDS-PAGE was performed using 12.5% polyacrylamide and stained by Coomassie Brilliant Blue; lane 1, 3 μg of V-ATPase; lane 2, 1 μg of VO.
V-ATPase Isoform

The role of the 116-kDa subunit in pump function remains to be elucidated. It appears to be an essential component of pumps from mammalian cells, and it is also present in pumps prepared from other sources (32, 33). In yeast, disruption of the genes encoding the two isoforms of this subunit resulted in conditional lethality (20, 21). All isoforms of 116-kDa subunit from mammalian cells have a strikingly similar structure, with a hydrophilic amino-terminal half and a hydrophobic carboxyl-terminal half that contains 6–8 transmembrane regions. Further analysis indicates that the mammalian isoforms share about 50% overall identity. The predicted membrane-spanning regions are even more conserved, with approximately 75% identity (Fig. 1), perhaps reflecting that these sectors participate in an essential, constitutive function such as transmembranous proton flow. The hydrophilic domains of these isoforms share about 25% identity, but all contain more than 30% charged residues.

An interesting aspect of 116-kDa isoforms is their differential expression in tissues. Isoform \( a_1 \) is highly enriched in brain (16, 17), and \( a_2 \) is more abundant in lung, kidney and spleen (Fig. 2), although both of them are present in most tissues. This may reflect organelle-specific distribution of isoforms within cells or cell-specific distribution within organs. An additional isoform, O\(_{1,16}\)-116 kDa, (which we term \( a_3 \)) was cloned by screening of a human osteoclastoma cDNA library and has been claimed to be specific to human osteoclastomas (18). However, its association with V-ATPases needs to be confirmed, and its distribution needs further investigation. These differential expressions of 116-kDa isoforms may provide very important clues in investigations of the subunit in pump function. A more detailed cytochemical analysis addressing this issue is under way in our laboratory.

Comparison of the sequences of the 116-kDa protein from bovine brain (\( a_1 \)) with that of its homologue from lung (\( a_2 \)), demonstrates the similarity of the two proteins and, at the same time, indicates that these two proteins must have arisen from separate genes. In fact, analysis of the predicted primary structures of all 116-kDa homologues from vertebrates indicates that three separate genes encode forms of these proteins. Supportive of this notion are the findings that multiple separate genes encode 116-kDa isoforms in \( C.\) \( elegans \) and yeast (20, 21). At present, the reasons for this extreme level of diversity of this subunit is unknown. However, we speculate that this diversity is related to the differential targeting and regulation of V pumps within eukaryotic cells.

It is, we believe, highly significant that the \( V_0 \) component of the proton pump of lung is found in a high molar ratio relative to the \( V_1 \) component, as compared with the \( V_1 \) \( V_0 \) constituents of bovine brain proton pump. It is possible that the stability of the \( V_1 \) \( V_0 \) complex prepared from clathrin-coated vesicles of bovine brain reflects the inherent stability of the complex in vivo. Thus, proton pumps localized to the constitutive endocytic pathway may not undergo dissociation into separate \( V_1 \) and \( V_0 \) domains, as has been described as an important regulatory mechanism for the V pumps of \( Saccharomyces\) \( cerevisiae \) and \( Manduca\) \( sexta \). We are currently engaged in localizing the \( a_2 \) isoform of lung to determine whether it is present in regulated secretory compartments within the epithelia of this tissue. In such a setting, it is possible that the \( V_1 \) and \( V_0 \) components undergo dissociation as a regulatory phenomenon and that this ability of the pump to dissociate into its two components is reflected at the biochemical level as an inherent instability in the \( V_1 \) \( V_0 \) complex.

In order to establish the association of cloned cDNA with V-ATPases, we have purified a vacuolar proton channel (\( V_0 \)) containing the \( a_2 \) isoform from bovine lung. This \( V_0 \) conducts proton flow after acid activation (Fig. 6) and pumps protons across the membrane after assembly with catalytic sector and SFD (Fig. 7), suggesting that isoform \( a_2 \) is indeed part of the V-ATPase.

A mouse form of the \( a_2 \) isoform was previously characterized molecularly in experiments designed to identify soluble immune regulatory factors (19). By structural predictions, neither the mouse nor the bovine form of this protein is soluble, and biochemical manipulations of the 116-kDa protein require detergents in our hands. Although we do not exclude the possi-
bility that the 116-kDa a2 isoform (or indeed any pump component) may play a role in eukaryotic cell functions beyond acidification, the experiments of this study indicate that the a2 isoform is present in V0 fractions that can catalyze proton flow. In fact, it is possible that isoforms of the 116-kDa subunit may play a role in T cell activation through its function as an essential component of the proton pump. It is notable in this regard that inhibition of V pump function in T cells by bafilomycin blocks antigen processing (34).

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