

Reconstitution of the Recombinant 70-kDa Subunit of the Clathrin-coated Vesicle H⁺ ATPase*

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Vacuolar-type proton pumps are complex hetero-oligomers. When dissociated into subcomplexes and subunits, the partial reactions of ATP hydrolysis and transmembranous proton flow can be assigned to isolated domains. Data suggest that the molecular site of ATP hydrolysis resides within the 70-kDa subunit but that ATPase activity likely requires at least three additional subunits of 58, 40, and 33 kDa (Xie, X.-S., and Stone, D. K. (1988) *J. Biol. Chem.* 263, 9859-9867). We have now cloned and sequenced the 70-kDa subunit from bovine brain and have expressed the protein in insect Sf9 (*Spodoptera frugiperda*) cells with a recombinant baculovirus. When purified, the protein has no significant ATPase activity but can be photoaffinity labeled with [α^{32} P]ATP and UV irradiation with an apparent K_d of 35 μ M. When reconstituted with biochemically prepared 58-, 40-, and 33-kDa polypeptides, the recombinant 70-kDa subunit restores Ca²⁺-activated ATP hydrolysis to a specific activity of 0.6 μ mol P_i/mg protein⁻¹·min⁻¹, thus demonstrating that ATP hydrolysis in vacuolar-type proton pumps is dependent upon both the 70-kDa subunit as well as multi-subunit interactions.

Vacuolar-type proton-translocating ATPases are responsible for the acidification of numerous cellular organelles, including clathrin-coated vesicles, endosomes, and lysosomes of mammalian cells, and vacuolar compartments of plant, yeast, and fungal cells (1, 2). Recently, V-type pumps have been identified in methanogenic bacteria (3) and eubacteria (4). In addition, vacuolar-type pumps are found in the plasma membranes of renal epithelial cells and osteoclasts, where they participate in urinary acidification (5) and bone reabsorption (6), respectively. Structurally, these proton pumps are complex hetero-oligomers with molecular masses of greater than 500 kDa. At least eight polypeptides are candidate subunits of the V-type proton-translocating ATPase of clathrin-coated vesicles of bovine brain; by SDS-PAGE,¹ these appear to have molecular masses of 116, 70,

58, 40, 38, 34, 33, and 17 kDa (7). Definition of the roles of these putative subunits is an ongoing aim of our work. Given the marked phylogenetic conservation of V-type pumps, structure-function analysis of the clathrin-coated vesicle proton pump is likely relevant to all V-type H⁺ ATPases.

To date, three functional domains of the clathrin-coated vesicle proton-translocating ATPase have been defined. These are an ATP hydrolytic sector, V_C, which contains (at least) the 70-, 58-, 40-, and 33-kDa subunits (8, 9); a transmembranous proton pore, V_B, consisting of the 116-, 39-, and 17-kDa subunits (10, 11); and a dissociable regulatory element (12).

Nucleotide labeling experiments (13, 14) and analysis of predicted primary structure (3, 4, 15-20) indicate that the 70-kDa polypeptide (A subunit) is the molecular site of ATP hydrolysis. The 58-kDa component (B subunit) has also been labeled with ATP analogues (21). Based upon these observations, a view has emerged that ATP hydrolysis of V-type pumps resembles that of F₁F₀-ATPases/synthetases and that subunit A (70-kDa) of the V-type pump is analogous to the β subunit of F₁.

From a functional standpoint, ATP hydrolysis catalyzed by the V-type proton pumps is considerably more complicated. Previously, we dissociated purified proton pump of clathrin-coated vesicles and partially resolved a collection of subcomplexes and dissociated pump subunits (8). This treatment resulted in a marked functional change in ATPase activity; whereas native enzyme, which is reconstitutively active, can hydrolyze ATP in the presence of Mg²⁺ or Ca²⁺, dissociated enzyme could not catalyze proton pumping after reconstitution into liposomes and could support ATP hydrolysis only in the presence of calcium.² Further dissociation of the pump yielded components that could not catalyze significant ATP hydrolysis (in the presence of Mg²⁺ or Ca²⁺), but Ca²⁺-activated ATPase activity was restored by the reconstitution of several of these isolated components. Although the resolution of subunits was incomplete, these studies suggested that calcium-activated ATP hydrolysis catalyzed by V_C required at least four polypeptides of 70, 58, 40, and 33 kDa (8, 9).

Attempts to further define, through a strictly biochemical approach, the minimal polypeptide requirement(s) for Ca²⁺-activated ATP hydrolysis have not been successful, due in large part to limited starting material (*i.e.* purified proton pump). As an alternative strategy, we are attempting to reconstitute all functional domains of the clathrin-coated vesicle proton pump through the use of recombinant subunits. In recent studies, we

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¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethylmaleimide;

ide; V_B, the bafilomycin-sensitive proton channel of the clathrin-coated vesicle proton pump; V_C, the dissociated catalytic domain of the clathrin-coated vesicle proton pump, which hydrolyzes CaATP.

² Recently, we established the relevance of this Ca²⁺-activated ATPase activity to proton pump function with the demonstration that reassembly of V_C, V_B, and a 50-57-kDa heterodimer restores Mg²⁺-activated ATPase activity and renders the enzyme capable of supporting proton pumping when reconstituted into liposomes (9).

have reconstituted purified recombinant 33-kDa (22) and 40-kDa (23) polypeptides to biochemically prepared subcomplexes, which were deficient in these components, and have demonstrated that both are genuine subunits essential for Ca^{2+} -activated ATP hydrolysis.

In this report, we show that the purified recombinant 70-kDa subunit lacks ATP-hydrolytic activity but can be labeled with [α - ^{32}P]ATP and UV irradiation. When reconstituted to biochemically prepared 58-, 40-, and 33-kDa polypeptides, the 70-kDa subunit restores ATPase activity to a specific activity of 0.6 $\mu\text{mol P}_i/\text{mg protein}^{-1}\cdot\text{min}^{-1}$, thus demonstrating that catalysis in vacuolar pumps has multiple subunit requirements.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones

A bovine brain cDNA library (insert size, 2.0–4.4 kilobases), transfected into *Escherichia coli* (strain BB4), was screened with a deoxyinosine-containing oligonucleotide (ACIGGCTICCCCTTCGTGAGC-TIGGICCGIATTTATGGGIGCIATTTTGATGG), which corresponded to the peptide sequence T229 (TGLPLSVELGPGIMGAIFDG) previously reported (24). Screening of 5×10^5 plaques was performed using a double-lift procedure wherein plaques were transferred to the plaque lift membranes for 1–1.5 min in the first lift and for 5 min in the second lift. The membranes were then prehybridized for 4 h at 68 °C in a solution containing 6 × SSC, 5 × Denhardt's solution, and 0.1 mg/ml sheared salmon sperm DNA. Positive plaques were identified by hybridization with 5'- ^{32}P -labeled oligonucleotides, which were added at a concentration of 5–10 × 10^5 cpm/ml of hybridization solution. Hybridization was performed at 54 °C for 16 h. The membranes were washed 3 times for 10 min for each cycle at room temperature in 6 × SSC, once for 2 min in 6 × SSC at 54 °C, and then were exposed to film for 12–48 h at –70 °C with intensifying screens. Duplicate positive clones were cored and rescreened through one or more cycles until purified colonies were obtained (25).

Subcloning and DNA Sequencing

Inserts of all positive clones were subcloned into Bluescript with helper phage R408 or VCSM13. Plasmid DNA was prepared by alkaline lysis (25), and DNA sequencing was carried out by the dideoxy-termination method (26) using single-stranded and/or double-stranded DNA as template. Single-stranded DNA was recovered from Bluescript through the use of M13 helper phage VCSM13. The cDNA clones were fully sequenced in both orientations. Data base searches were performed using PC/Gene-based programs.

Expression of the 70-kDa Subunit of Bovine Brain H^+ ATPase

The coding region for the 70-kDa subunit of the H^+ ATPase was amplified by polymerase chain reaction using cloned cDNA as a template, and two oligonucleotides, 5'-GCGCATATGATGATTCTCC-AAGTCCCC-3' and 5'-GGAGAGATCTCAGAATTCTAATCTTCAAG-GCTACGG-3', contained *Nde*I and *Bgl*II restriction sites and initiator and stop codons at the 5'-ends to enable cloning into the expression vector p^{ET3a} (27, 28). Bacterial expression vector p^{ET3a70} was constructed by replacing the *Nde*I–*Bam*HI fragment of plasmid p^{ET3a} with the polymerase chain reaction product after digestion with *Nde*I and *Bgl*II. The recombinant plasmid was characterized by hybridization, restriction enzyme mapping, and DNA sequencing.

The bacterial expression vector p^{ET3a70} was digested with *Bgl*II and *Eco*RI. The resultant 1.9-kilobase fragment, which contained the entire coding region of the 70-kDa subunit, was cloned into the *Bgl*II and *Eco*RI sites of the baculovirus expression vector p^{VL1392}. The resultant plasmid, p^{VL1392-70}, was characterized by restriction enzyme mapping and partial DNA sequencing. Plasmid DNA was prepared with alkaline lysis and was purified by two cesium chloride gradient centrifugation steps (25).

Sf9 (*Spodoptera frugiperda*) ovary cells were propagated in monolayer or in suspension culture at 27 °C in Grace's or IPL-41 medium supplemented with 10% fetal calf serum as described (22, 29). Recombinant baculovirus was generated by cotransfection of Sf9 cells with the expression vector p^{VL1392-70} and linearized AcRP23-LacZ viral DNA by the Lipofectin method (22). Positive viral clones were isolated by plaque assay and identified by their ability to direct the expression of 70-kDa protein as determined by SDS-PAGE and Western blot analysis.

For expression and production of the 70-kDa subunit, Sf9 cells (1000 ml cells at 2×10^6 cells/ml medium) were grown in suspension culture

at 27 °C, infected with pure recombinant baculovirus for 48 h, and harvested by centrifugation at $4000 \times g$ for 15 min at 4 °C. The pellet was resuspended in 50 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride and sonicated for 1 min on ice. The preparation was examined under the microscope to achieve efficient cell lysis with minimal sonication. The lysate was then centrifuged at 40,000 rpm for 45 min at 4 °C with a Beckman 70 Ti rotor. The recombinant 70-kDa subunit was purified as follows.

Step 1. Hydroxylapatite Chromatography—The supernatant (50 ml) was loaded on a hydroxylapatite chromatography column (50 ml), which had been pre-equilibrated with lysis buffer. The column was eluted with a continuous 500-ml sodium phosphate gradient (0–400 mM P_i , pH 7) prepared in lysis buffer.

Step 2. $(\text{NH}_4)_2\text{SO}_4$ Fractionation—Fractions containing the recombinant 70-kDa subunit (as determined by SDS-PAGE) were pooled (40 ml), and $(\text{NH}_4)_2\text{SO}_4$ was added to a final saturation of 25%. After centrifugation at $100,000 \times g$ for 30 min, the pellet was discarded, and the $(\text{NH}_4)_2\text{SO}_4$ saturation of the supernatant was brought to 60%. After repeat centrifugation as above, the pellet was saved.

Step 3. Glycerol Gradient Centrifugation—The final $(\text{NH}_4)_2\text{SO}_4$ pellet was dissolved in 1 ml of buffer A (10 mM Tris-MES, pH 7.0, 5 mM dithiothreitol, 0.5 mM EDTA, 0.02% polyoxyethylene 9-lauryl ether), and the solution was loaded on a continuous glycerol gradient (13.5 ml, 10–30%) prepared in buffer A. The gradient was centrifuged at $170,000 \times g$ for 24 h, and fractions were collected from the bottom after piercing the tube.

Step 4. High Pressure Liquid Chromatography—Fractions containing the 70-kDa subunit, as determined by SDS-PAGE, were pooled (5 ml) and were loaded in 0.75-ml aliquots on three Beckman Spherogel 3,000 TSK columns (7.5×20 mm) connected in series. A solution of 0.1 M NaCl and 0.5 mM EDTA was used as an eluant with a flow rate of 0.5 ml/min and continuous monitoring at λ_{280} . 30 1-ml fractions were collected per run, and molecular mass calibration was performed using standards ranging from tryptophan to thyroglobulin. This procedure yielded about 5 mg of purified 70-kDa subunit, which was used for reconstitution and ATP labeling studies described below.

Reconstitution and Measurement of ATPase Activity

The proton pump of clathrin-coated vesicles was purified (7), dissociated by urea treatment, and fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation; subsequent glycerol gradient centrifugation yielded dissociated 58-, 40-, and 33-kDa polypeptides as reported (8). Optimal reconstitution of Ca^{2+} -activated ATPase activity was achieved by incubating these polypeptides with the recombinant 70-kDa subunit at a mass ratio of 1 for 10 min at room temperature in the presence of 2.5 μg of phosphatidylserine. ATPase activity was measured by the liberation of $^{32}\text{P}_i$ from [γ - ^{32}P]ATP. The assay solution consisted of 50 mM Tris-MES, pH 7.0, 30 mM KCl, 3 mM CaCl_2 , and 2 mM [γ - ^{32}P]ATP (200–400 cpm/nmol). Reactions were initiated by addition of 100 μl of assay solution. After incubation at 37 °C for 30 min, the reactions were terminated by the addition of 1.0 ml of 1.25 N perchloric acid, and liberated $^{32}\text{P}_i$ was extracted and measured as described (30).

[α - ^{32}P]ATP Labeling

Photoaffinity labeling of the recombinant 70-kDa subunit was performed essentially as described (31–33). A reaction mixture (20 μl) containing purified recombinant 70-kDa subunit (0.2 μg), 20 mM Tris-MES (pH 7.0), 10% glycerol (v/v), [α - ^{32}P]ATP, and other components (as noted in the legends to the figures) was incubated on ice for 5 min and then irradiated for 8 min on ice at a distance of 35 mm from the UV source (model R-52G, UVP, Inc., San Gabriel, CA). The reaction was stopped by the addition of 2.2 μl of concentrated SDS sample buffer containing 7.5% SDS and 0.3 M Tris-HCl (pH 6.8). After SDS-PAGE (34), the gel was dried and autoradiographed on Kodak X-OMAT film at –80 °C for 24–48 h. Incorporation of [α - ^{32}P]ATP was determined by scanning autoradiograms with an image Quan densitometer (Molecular Dynamics 300A computing densitometer) to determine the relative ^{32}P incorporation, quantified as absorbance area on the basis of whole band analysis. Exposure times of the film were adjusted to ensure that the image would fall well within the linear range of detection.

Miscellaneous

Western blot analysis was performed using a previously described anti-70-kDa antibody at a 1:2000 dilution under reported conditions (6). Protein was determined by the Amido Schwarz method (35). A bovine brain cDNA library in λ_{ZAP} was the kind gift of Dr. Richard A. F. Dixon (The University of Texas Health Science Center at Houston). The

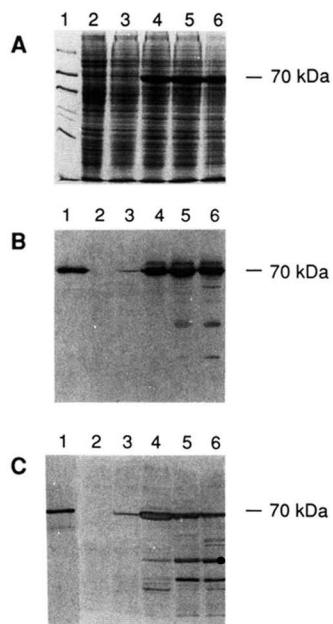


FIG. 1. SDS-PAGE and Western blot analysis of the 70-kDa polypeptide expressed in Sf9 cells transfected with recombinant baculovirus. Lysates of Sf9 cells were subjected to SDS-PAGE (A) or Western blot analysis (B and C) as described under "Experimental Procedures." A and B illustrate total cellular lysates, and C illustrates the soluble portion of cell lysates, defined as the supernatant obtained after centrifuging the total cellular lysate at $150,000 \times g$ for 1 h. Lane 1, purified proton pump from bovine brain; lane 2, non-transfected Sf9 cells; lanes 3–6, Sf9 cells transfected with recombinant baculovirus for 24, 48, 72, or 96 h, respectively.

sources of additional reagents used in this study have been previously identified (7, 8, 11, 22–24).

RESULTS

Isolation and Identification of cDNAs Encoding the 70-kDa Subunit of Bovine Clathrin-coated Vesicle Proton ATPase—Screening of 5×10^5 recombinant clones from a bovine brain cDNA library resulted in the identification of four positive clones of interest, designated $p^{13-1(1)}$, $p^{13-2-3(1)}$, $p^{25-1(1)}$, and $p^{29-1(1)}$. Among these clones, $p^{29-1(1)}$ and $p^{13-2-3(1)}$ contained the entire coding region of the 70-kDa subunit of the vacuolar H^+ ATPase. The cDNA sequence of the clone $p^{29-1(1)}$ was determined in both orientations, and partial sequences of the other three clones were also analyzed. All four cDNAs had identical sequences at overlapping regions. The complete sequence of $p^{29-1(1)}$ (data not shown) contains 2541 base pairs, which includes the entire coding region. The open reading frame consists of 1851 nucleotides, encoding 617 amino acid residues, for a calculated molecular mass of 68,344 Da. The predicted sequence of amino acids includes all nine peptides that had been determined by direct sequencing of tryptic peptides derived from purified 70-kDa subunit (24), thus confirming that the cloned cDNAs encode the 70-kDa proton pump component. Using a *Pst*I fragment of cDNA clone $p^{29-1(1)}$ as a probe, Northern blot analysis of poly(A)⁺ RNA from bovine brain and total RNA from bovine brain, heart, kidney, liver, and spleen revealed in all instances a single mRNA species of approximately 4.5 kilobases (data not shown).

Expression of the 70-kDa Subunit in Sf9 Cells—Sf9 cells in suspension culture were infected with recombinant baculovirus as described under "Experimental Procedures." As shown in Fig. 1, insect cell lysates were analyzed by SDS-PAGE (panel A) and Western blot analysis (panel B), revealing a distinct band of about 70 kDa (lanes 3–6), which is absent in the cell lysate from uninfected cells (lane 2). The recombinant 70-kDa

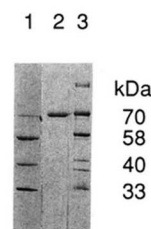


FIG. 2. Biochemically prepared proton pump and polypeptides and purified recombinant 70-kDa polypeptide. SDS-PAGE was performed as described under "Experimental Procedures." Lane 1, purified ATP catalytic core, depleted of the 70-kDa subunit; lane 2, purified recombinant 70-kDa polypeptide; lane 3, purified clathrin-coated vesicle proton pump from bovine brain.

protein was present as early as 24 h postinfection as revealed by Western blot analysis (panel B, lane 3) and was readily detected on a Coomassie Blue-stained gel 48 h postinfection (panel A, lane 4). The production of the recombinant 70-kDa protein reached maximal level 72–96 h postinfection (panel A, lanes 5 and 6), constituting about 5% of the total cellular protein.

Purification of the Recombinant 70-kDa Subunit from Infected Sf9 Cells—Although maximal expression of the 70-kDa subunit occurred 72–96 h postinfection, recombinant protein was largely aggregated at this point and was not useful for purification. Western blot analysis of the soluble fraction of Sf9 lysates (Fig. 1, Panel C) demonstrated that 48 h postinfection of Sf9 cells (lane 4) was optimal for harvesting soluble protein under non-denaturing conditions. Fig. 2 (lane 2) illustrates recombinant 70-kDa protein purified from Sf9 cells 48 h postinfection as described under "Experimental Procedures."

ATP Binding Assay of the Recombinant 70-kDa Protein—Since the 70-kDa subunit contains a consensus sequence for ATP binding, ATP labeling of the recombinant protein was attempted using [α - 32 P]ATP and ultraviolet irradiation. As shown in Fig. 3, [α - 32 P]ATP labeling was modulated by divalent cations; labeling was more intense with Mg^{2+} (lane 2) than with Ca^{2+} (lane 1) and was largely eliminated by the omission of divalent cations (lane 3). In separate experiments, we have determined that the low level of labeling observed in lane 3 occurs even in the presence of EDTA and EGTA (data not shown). In addition, the ATP labeling was largely eliminated by 10 mM NEM (lane 4), implying a possible competition between NEM and ATP at the ATP binding site of the 70-kDa subunit. In contrast, bafilomycin, a potent and specific inhibitor of V-type H^+ ATPases (36), did not inhibit ATP labeling even at 10 μ M, a concentration about 1,000-fold greater than that required to inhibit vacuolar H^+ pumps (data not shown).

Photoaffinity labeling of the 70-kDa polypeptide with [α - 32 P]ATP was a saturable process; 80–100 μ M ATP was sufficient to saturate photolabeling (Fig. 4). The apparent K_{dATP} determined from double reciprocal plots of the response curve shown in Fig. 4 was 35 μ M.

Reconstitution of ATP Hydrolysis—Proton-translocating ATPase, purified from clathrin-coated vesicles of bovine brain, was partially dissociated with 3.1 M urea. After ammonium sulfate precipitation and glycerol gradient centrifugation, a fraction, which had been largely depleted of the 70-kDa component, was obtained, as shown in Fig. 2 (lane 1). Densitometric analysis of this fraction revealed that it was 94% depleted of the 70-kDa subunit as compared with native enzyme. This fraction was assessed, with or without the recombinant 70-kDa subunit, for its capacity to support ATP hydrolysis. As shown in Table I, neither purified recombinant 70-kDa polypeptide nor the 70-kDa-depleted fraction could catalyze significant ATP hydrolysis. However, the combination of these components yields NEM-sensitive, Ca^{2+} -activated ATP hydrolysis with a specific

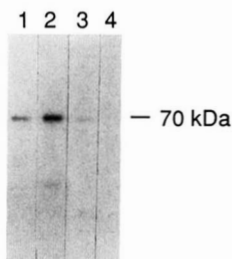


FIG. 3. Photoaffinity labeling of the recombinant 70-kDa subunit with $[\alpha\text{-}^{32}\text{p}]\text{ATP}$. Reaction mixtures (20 μl) containing 0.2 μg of the 70-kDa protein and 3.3 pmol of $[\alpha\text{-}^{32}\text{p}]\text{ATP}$ (3,000 Ci/mmol) were irradiated for 8 min on ice, followed by SDS-PAGE and autoradiography, as described under "Experimental Procedures." Specific reaction conditions were as follows: lane 1, 3 mM CaCl_2 ; lane 2, 3 mM MgCl_2 ; lane 3, no divalent cation; lane 4, 3 mM MgCl_2 and 10 mM NEM.

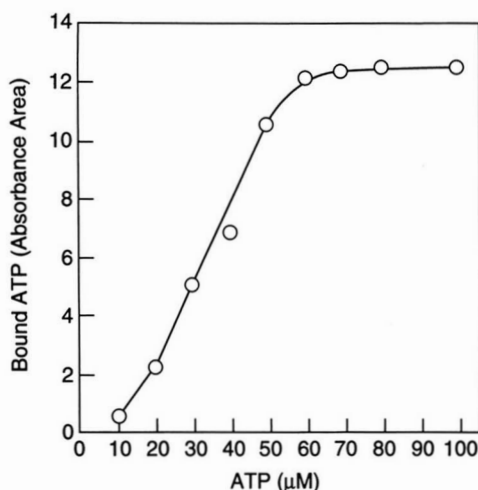


FIG. 4. Kinetics of photoincorporation of $[\alpha\text{-}^{32}\text{p}]\text{ATP}$ into recombinant 70-kDa polypeptide. 70-kDa polypeptide was UV-irradiated in the presence of 3 mM MgCl_2 and the indicated concentration of $[\alpha\text{-}^{32}\text{p}]\text{ATP}$ (0.25 $\mu\text{Ci}/\mu\text{M}$ ATP) and separated by SDS-PAGE. Incorporation of $[\alpha\text{-}^{32}\text{p}]\text{ATP}$ into 70-kDa polypeptide was determined from densitometric scans of the autoradiograms.

TABLE I
Reconstitution of the recombinant 70-kDa subunit of the clathrin-coated vesicle (H^+) ATPase

ATPase activities were measured as described under "Experimental Procedures" using 1.0 μg of purified recombinant 70-kDa protein, 1.0 μg (total) of the 58-, 40-, and 33-kDa polypeptides (shown in Fig. 4, lane 1), and 5 mM NEM as indicated.

Polypeptides	Ca^{2+} -ATPase activity $\text{nmol Pi}\cdot\text{min}^{-1}\cdot\text{assay}^{-1}$
70-kDa	<0.01
58-, 40-, and 33-kDa	<0.01
58-, 40-, and 33-kDa + 70-kDa	1.2
58-, 40-, and 33-kDa + 70-kDa + NEM	0.023

activity of about 0.6 $\mu\text{mol P}/\text{mg protein}/\text{min}$. The K_m for ATP was 95 μM under the conditions described in the legend to Fig. 5. This K_m value is the same as that achieved with the native catalytic sector of V-type ATPase.³

Fig. 6 demonstrates the influence of various concentrations of recombinant 70-kDa polypeptide on the reconstitution activity of ATP hydrolysis. As shown, restoration of Ca^{2+} -activated ATPase is a saturable function of the concentration of the recombinant 70-kDa subunit.

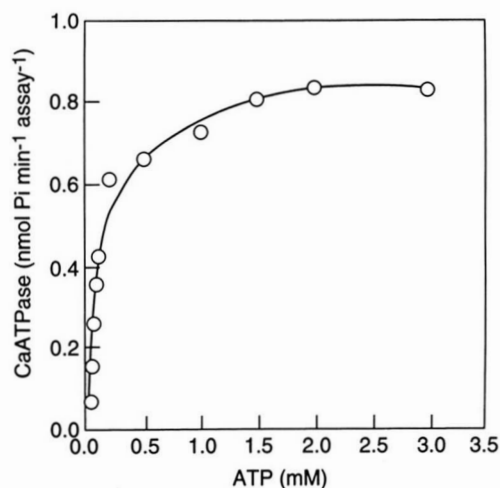


FIG. 5. Effect of ATP concentration on reconstitution of Ca^{2+} -activated ATP hydrolysis. ATPase activities were measured as described under "Experimental Procedures" using 1 μg of a fraction containing 33-, 40-, and 58-kDa polypeptide, 1 μg of 70-kDa polypeptide, and indicated ATP concentrations.

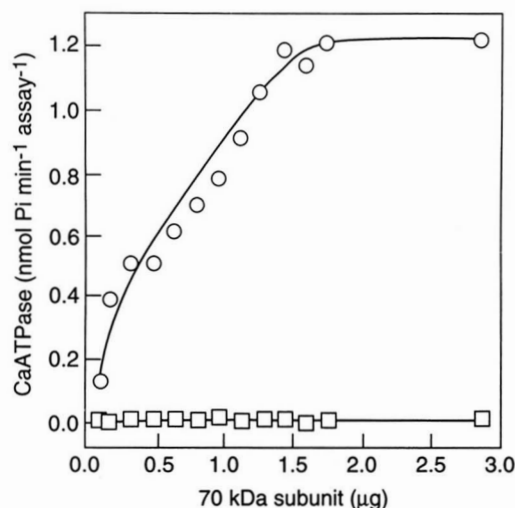


FIG. 6. Effect of 70-kDa polypeptide concentration on reconstitution of Ca^{2+} -activated ATP hydrolysis. ATP activities were measured as described under "Experimental Procedures" using 1 μg of the 33-, 40-, and 58-kDa polypeptide mixture and designated concentrations of 70-kDa polypeptide (\circ) or 70-kDa protein alone (\square).

DISCUSSION

Functional analysis of the subunits of the clathrin-coated vesicle proton pump through the approach of resolution and reconstitution requires large amounts of pure components. For this reason, we have cloned, expressed, and purified the recombinant 70-kDa subunit in a reconstitutively active form. The cloned cDNA encodes 617 amino acids in a sequence that is identical to that of the 70-kDa subunit of bovine adrenal medulla (37) but differs from a previously reported sequence of the bovine brain 70-kDa subunit (18) at amino acid 91. Our translated sequence from cDNA as well as direct peptide sequencing (24) indicate that this residue is glycine; this view is supported by the finding that all previously reported sequences have a glycine residue at position 91 (15–17, 19, 20, 37). An additional difference between our cDNA sequence and that previously reported for bovine brain (18) lies within the 3'-untranslated region. Although our 3'-untranslated region of cDNA is incomplete, it is identical to the 3'-untranslated region of a bovine adrenal medulla clone (37) but radically differs from that previously reported (18). It is most likely that the sequence of the

³ D. K. Stone and X.-S. Xie, unpublished data.

3'-untranslated region of the latter owes to a library construction artifact, which derives from the introduction of an additional *EcoRI* site.

Irrespective of these issues, our purpose in cloning the 70-kDa subunit and the importance of this study lies in the reconstitution of purified recombinant 70-kDa subunit. Previously, we have successfully purified and renatured recombinant 33- and 40-kDa subunits that were expressed in *E. coli* (22, 23). Initially, we pursued a similar approach with the 70-kDa subunit but were unable to renature the purified protein to yield a reconstitutively active component. Consequently, we adopted an expression and purification protocol using Sf9 cells. Success with this system required that the incubation period with recombinant baculovirus be titrated, not against total cellular production of the protein but rather against the expression of soluble recombinant A subunit, as shown in Fig. 1 (panel C). Close inspection of this panel reveals that incubation periods longer than 48 h (lanes 5 and 6) resulted in a real decrease in the soluble portion of the recombinant 70-kDa component as well as proteolysis, as evidenced by the appearance of multiple sub-70-kDa bands that reacted with anti-70-kDa antibody.

As shown in Figs. 2 and 3 and Table I, purified 70-kDa subunit could not only support nucleotide binding but also resulted in a substantial Ca^{2+} -activated ATPase activity when reconstituted to a biochemically generated, 70-kDa depleted fraction. It is of note that the recombinant 70-kDa subunit as well as the 58-, 40-, and 33-kDa polypeptide fractions have negligible ATPase activity. Thus, our data indicate that the 70-kDa subunit is the actual site of ATP binding, as indicated by nucleotide labeling, but that it alone is unable to support Ca^{2+} -activated ATP hydrolysis (Fig. 6). Although preparations of recombinant 70-kDa polypeptide consistently support minimal Mg^{2+} -activated ATPase activity ($0.004 \mu\text{mol P}_i/\text{mg protein}^{-1}\cdot\text{min}^{-1}$), we are concerned that this Mg^{2+} -activated ATPase activity may be caused by low level contamination present in the 70-kDa fraction. It is also possible that the 70-kDa polypeptide does have minimal catalytic activity, as does the isolated β subunit of *E. coli* F_1 , which has roughly 1/10,000 the catalytic rate of intact F_1 (38). Site-directed mutagenesis studies designed to examine this issue and the relationship of nucleotide binding to hydrolysis in the 70-kDa subunit are in progress.

In addition to demonstrating the essential nature of this subunit in pump function, this work underscores our point that ATP hydrolysis requires multiple components. These results are not to be interpreted that the actual ATP hydrolysis site spans four different subunits. All evidence indicates that the 70-kDa subunit is the likely site of catalysis (1, 2), and our data are reconciled with this point in the proposition that additional components (58, 40, and 33 kDa) are necessary to render the 70-kDa subunit catalytically active. In this respect, the V-type ATPase thus resembles F_1 ATPases, where the β subunit, a nucleotide binding protein, is thought to be the actual catalytic site, and other subunits (α , γ , δ , ϵ) are necessary for maximal enzymatic activity (39, 40).

It is our goal to reconstitute the catalytic sector of the bovine brain proton pump from purified recombinant subunits. This approach, necessitated by the difficulty in obtaining sufficient amounts of biochemically purified subunits to perform definitive experiments, has the advantages of allowing for the unequivocal identification of subunits as well as their production in large scale. The latter, in turn, permits detailed biochemical

analysis of wild-type and mutagenized subunit function and interaction (which is not possible by a strict biochemical approach), as well as the potential for crystallization of single subunits and/or the functional catalytic sector. With the previous reconstitution of purified recombinant 33- (22) and 40-kDa (23) components, the reconstitution of the 70-kDa described in this article, and our most recent reconstitution of purified recombinant 58-kDa subunit,⁴ we now turn our attention to these aims.

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