Subunit Composition and ATP Site Labeling of the Coated Vesicle Proton-Translocating Adenosinetriphosphatase†

Hiroyuki Arai,‡ Michael Berne, Geronimo Terres, Helen Terres, Karen Puopolo, and Michael Forgac*

Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received April 22, 1987; Revised Manuscript Received June 8, 1987

ABSTRACT: The partially purified proton-translocating adenosinetriphosphatase [(H^+)-ATPase] from clathrin-coated vesicles has been reported to contain eight polypeptides of molecular weights 15 000–116 000 [Xie, X. S., & Stone, D. K. (1986) *J. Biol. Chem. 261*, 2492–2495]. To determine whether these polypeptides form a single macromolecular complex, we have isolated three monoclonal antibodies which recognize the reconstitutively active (H^+)-ATPase in the native, detergent-solubilized state. All three monoclonal antibodies precipitate the same set of polypeptides from either the partially purified enzyme or the detergent-solubilized coated vesicle membrane proteins. The immunoprecipitated polypeptides have molecular weights of 100 000, 73 000, 58 000, 40 000, 38 000, 34 000, 33 000, 19 000, and 17 000. These results thus indicate that this set of polypeptides forms a single macromolecular complex and suggest that they correspond to subunits of the coated vesicle (H^+)-ATPase. To identify the ATP-hydrolytic subunit of the coated vesicle (H^+)-ATPase, the purified enzyme was reacted with *N*-ethylmaleimide (NEM) and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), both of which inhibit activity in an ATP-protectable manner. Labeling was carried out by using [^{3}H]NEM or [^{14}C]NBD-Cl, and the specificity of the reaction was increased by prelabeling of the protein with the nonradioactive reagents in the presence of ATP and by taking advantage of the nucleotide specificity of protection. The principal polypeptide labeled by both [^{3}H]NEM and [^{14}C]NBD-Cl had a molecular weight of 73 000. In addition, this protein was the only polypeptide whose labeling was significantly reduced in the presence of ATP. These results suggest that the 73 000-dalton polypeptide participates in ATP hydrolysis by the coated vesicle (H^+)-ATPase.

The coated vesicle proton pump (Forgac et al., 1983; Stone et al., 1983) belongs to a novel class of intracellular proton pumps which includes those present in endosomes (Galloway et al., 1983; Yamashiro et al., 1983), lysosomes (Schneider, 1981; Harikumar & Reeves, 1983), Golgi (Glickman et al., 1983; Zhang & Schneider, 1983), chromaffin granules (Cidon & Nelson, 1983; Dean et al., 1984), and the vacuolar membranes of plants (Wang & Sze, 1985), *Neurospora* (Bowman, 1983), and yeast (Uchida et al., 1985). These enzymes are all resistant to vanadate (which inhibits those pumps that undergo active-site phosphorylation) and oligomycin [which inhibits the coupling factor proton-translocating adenosinetriphosphatase ((H^+)-ATPases)[1]]. On the other hand, this class of intracellular proton pumps is sensitive to the alkylating agents NEM and NBD-Cl and the carboxyl reagent DCCD (Forgac & Cantley, 1984; Stone et al., 1983). In addition, proton transport by these enzymes is electrogenic, with charge compensation provided by an accompanying anion flux (Xie et al., 1983) rather than by countertransport of cations (Forgac & Cantley, 1984).

Although the polypeptide composition of this class of proton pumps has not yet been completely defined, structural information on a number of members of this class has begun to emerge. The (H^+)-ATPase from plant tonoplasts appears to have a relatively simple subunit composition, containing at least three principal polypeptides of molecular weight 72 000, 60 000, and 17 000 (Randall & Sze, 1986; Mandala & Taize, 1986). By contrast, a partially purified preparation of the coated vesicle (H^+)-ATPase has recently been reported to contain eight polypeptides of molecular weights 15 000–116 000 (Xie & Stone, 1986).

To address the question of whether the polypeptides present in the partially purified (H^+)-ATPase from coated vesicles form a single macromolecular complex, we have employed a series of monoclonal antibodies which recognize this enzyme in the native, detergent-solubilized form. In addition, we have carried out labeling studies using two ATP-protectable inhibitors of the coated vesicle pump, NEM and NBD-Cl, in order to identify the ATP-hydrolytic subunit of this enzyme.

Materials and Methods

Materials. Calf brains were obtained fresh from a local slaughterhouse. Phosphatidylcholine and phosphatidylserine were obtained as chloroform solutions from Avanti Polar Lipids, Inc., and stored at −20 °C. NEM, $C_{12}E_9$, cholesterol, cholic acid, Na_2ATP (grade II), and ouabain were purchased from Sigma Chemical Co., and both cholic acid and cholesterol were recrystallized prior to use. NBD-Cl and acridine orange were obtained from Eastman Kodak. [^{3}H]NEM (40 Ci/mmol) and [γ-^{32}P]ATP were purchased from New England

† This work was supported in part by National Institutes of Health Grant GM 34478. Fluorescence facilities were provided through National Institutes of Health Grant P30 DK 34928.

* Correspondence should be addressed to this author. He is an American Heart Association Established Investigator.

‡ American Heart Association Postdoctoral Fellow.

[1] Abbreviations: (H^+)-ATPase, proton-translocating adenosinetriphosphatase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NEM, *N*-ethylmaleimide; $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; SDS, sodium dodecyl sulfate; DCCD, dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

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Nuclear, [^{14}C]NBD-Cl (109 mCi/mmol) was purchased from Research Products International, and ^{125}I-Bolton–Hunter reagent was obtained from ICN.

Unless otherwise indicated, experiments were carried out in solubilization buffer containing 50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 10% glycerol, and 0.2 mM EGTA.

Assays. ATPase activity was measured by a continuous spectrophotometric assay (Forgac et al., 1983) or by release of [^{32}P]P_i from [γ-^{32}P]ATP as previously described (Goldin, 1977). Activity was measured in solubilization buffer containing 0.02% $C_{12}E_9$, 8 μg of phosphatidylcholine/mL, and 4 μg of phosphatidylserine/mL to maintain the protein in a soluble state. Proton transport in reconstituted vesicles was measured by acridine orange fluorescence quenching using a Perkin-Elmer LS-5 spectrofluorometer as previously described (Forgac & Berne, 1986). Protein concentration was determined either by the method of Lowry et al. (1951) or, for samples containing $C_{12}E_9$, by the method of Schaffman and Weissman (1973).

Partial Purification of the Coated Vesicle (H^+)-ATPase. Clathrin-coated vesicles were prepared from calf brain as previously described (Forgac & Cantley, 1984). Vesicles were stripped of their clathrin coat by dilution (40-fold) into 5 mM Tris (pH 8.5), 150 mM sucrose, and 0.5 mM EDTA followed by incubation for 1 h at 23 °C and sedimentation for 1 h at 100000*g*. Stripped vesicles displayed a 10-fold higher specific activity for the (H^+)-ATPase relative to intact coated vesicles.

The (H^+)-ATPase was solubilized from stripped vesicles by incubation at a protein concentration of 0.5 mg of protein/mL with 0.5% $C_{12}E_9$ and 0.2 mg of phosphatidylcholine/mL in solubilization buffer at 23 °C for 5 min followed by 20 min on ice. The solubilized mixture was then sedimented for 1 h at 100000*g* and the supernatant removed and used as described below.

The $C_{12}E_9$-solubilized (H^+)-ATPase was purified by density gradient sedimentation by applying 0.5 mL of solubilized enzyme to 11-mL 15–30% glycerol gradients containing 0.02% $C_{12}E_9$ and 8 μg of phosphatidylcholine/mL. Sedimentation was carried out for 16 h at 38 000 rpm (175000*g*) in a Beckman SW 41 rotor using a Beckman L7-55 ultracentrifuge, and the gradients were fractionated from the bottom to give 18–20 fractions of 0.61 mL. The fractions were then assayed for ATPase activity as described above.

In the presence of glycerol, two peaks of ATPase activity are observed. The heavier peak, sedimenting as a complex of molecular weight 500 000 (Xie & Stone, 1986), contains all of the subunits required for reconstitution of proton transport (see below) and is the preparation used in the studies described. The specific activity of this partially purified enzyme was typically 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C, corresponding to a 100-fold purification relative to intact coated vesicles.

Reconstitution of the Coated Vesicle (H^+)-ATPase. The partially purified enzyme (prepared as described above) was reconstituted into phospholipid vesicles as follows. The peak fractions from the gradient were pooled and concentrated to 0.5 mL with a Centricon 10 microconcentrator. To the pooled fractions were then added the following detergent–phospholipid mixtures: 100 μL of 5% cholate, 4 mg of phosphatidylcholine/mL, 2 mg of phosphatidylserine/mL; 140 μL of 10% cholate, 4 mg of cholesterol/mL, 7 mg of phosphatidylcholine/mL. Following incubation for 5 min at 23 °C and 20 min on ice, the mixture was dialyzed for 3 days against four changes of 500 volumes of solubilization buffer using Spectrapor 2 dialysis tubing with a molecular weight cutoff of 12000–14000. ATP-dependent acidification of reconstituted vesicles was measured by using acridine orange (see above) in the presence of 3.3 μM valinomycin to prevent generation of a membrane potential during proton uptake.

Preparation and Screening of Monoclonal Antibodies. Vesicles (100 μg of protein) from which the clathrin coat had been dissociated (see above) were suspended in Freund's complete adjuvant and injected subcutaneously into female Balb/c mice. Mice were given booster injections intraperitoneally after 2, 3, and 5 weeks followed by a final injection after 5 months using antigen suspended in solubilization buffer. The sera were tested for the presence of antibodies which recognized the coated vesicle (H^+)-ATPase by the assays described below. Three days after the final injection, the spleens were removed, the dissociated cells fused with the mouse myeloma cell line P3X63Ag8.653 using poly(ethylene glycol), and the fused cells selected by growth in HAT media as previously described (Galfre & Milstein, 1981). After growth for 2 weeks in 96-well plates, the wells were screened by using the procedures described below.

Clones producing antibodies which recognized some protein in the partially purified enzyme were identified by ELISA as follows. Ninety-six well plates were coated with the partially purified enzyme followed by blocking with horse serum. The primary antibody, biotin-labeled goat anti-mouse IgG (Sigma), and avidin-conjugated horseradish peroxidase (Sigma) were added sequentially with incubation for 2 h at 37 °C and washing after each addition. Following the final washing, samples were incubated for 1 h at 23 °C with 0.4 mg of *o*-phenylenediamine/mL and 0.12% H_2O_2, and the absorbance at 490 nm was measured. The wells showing the most positive reaction by ELISA were then screened as described below.

Clones producing antibodies capable of immunoprecipitating the native, detergent-solubilized (H^+)-ATPase in an active state were identified as follows. Culture supernatants (100 μL) containing the antibody being tested or buffer was mixed with 100 μL of purified enzyme (0.3–0.4 μg of protein), and the mixture was incubated for 2 h on ice. Protein A–Sepharose CL-4B (75 μL of a 50% suspension; Pharmacia) was then added and the mixture shaken for 1 h at 4 °C followed by washing of the Sepharose particles by sedimentation for 2 min in a Beckman Model B microfuge using solubilization buffer containing 0.02% $C_{12}E_9$, 8 μg of phosphatidylcholine/mL, and 4 μg of phosphatidylserine/mL [detergent was maintained throughout the procedure at 0.02% to prevent aggregation of the (H^+)-ATPase]. The immunoprecipitated enzyme was then assayed for ATPase activity by addition of 200 μL of solubilization buffer containing 1 mM [γ-^{32}P]ATP (12 mCi/mmol), 5 mM $MgSO_4$, 2 mM potassium phosphate, 100 μM ouabain, and 0.2% $C_{12}E_9$ (with phospholipids in the same ratio to detergent). Following incubation of the mixture for 3 h at 23 °C with shaking, the Sepharose particles were removed by sedimentation for 1 min at 10000*g*, and an aliquot of the supernatant was assayed for the amount of [^{32}P]P_i released as previously described (Goldin, 1977). Clones testing positive by both ELISA and immunoprecipitation of (H^+)-ATPase activity were then subcloned and the culture supernatants retested.

Control antibodies were obtained (i.e., 2C-9H) which tested positive by ELISA using a crude mixture of coated vesicle membrane proteins but which failed to immunoprecipitate an active (H^+)-ATPase. Western blots of the partially purified enzyme using the positive and control monoclonals were carried out by using alkaline phosphatase conjugated goat anti-mouse IgG (Bio-Rad Immun-Blot assay kit 170-6511).

Immunoprecipitation of ^{125}I-Labeled (H^+)-ATPase. To identify the polypeptides immunoprecipitated by the monoclonal antibodies which recognized the native coated vesicle (H^+)-ATPase, the partially purified enzyme (1.2 μg of protein) was first labeled with ^{125}I-Bolton–Hunter reagent. The ^{125}I-labeled enzyme was then immunoprecipitated by using either positive or control antibodies and protein A–Sepharose as described above except that following the 1-h incubation with protein A–Sepharose and washing to remove unbound enzyme, the immunoprecipitated proteins were eluted from the Sepharose particles with Laemmli sample buffer, separated by SDS–polyacrylamide gel electrophoresis on 8% or 12% acrylamide gels, and identified by autoradiography as described below.

To identify the polypeptides immunoprecipitated from detergent-solubilized, coated vesicle membrane proteins, the clathrin coat was first dissociated, and the membrane proteins (50 μg of protein) were solubilized with $C_{12}E_9$ and labeled with ^{125}I-Bolton–Hunter reagent as described above. Immunoprecipitation of the (H^+)-ATPase from this material was carried out by using 300 000 cpm of ^{125}I-labeled protein.

Labeling of the Coated Vesicle (H^+)-ATPase with [^{3}H]-NEM and [^{14}C]NBD-Cl. Prior to being labeled with [^{3}H]-NEM, the partially purified enzyme was first reacted with nonradioactive NEM in the presence of ATP in order to reduce the number of nonspecific sites capable of reacting with [^{3}H]NEM. The partially purified (H^+)-ATPase (14 μg of protein) in 0.6 mL of solubilization buffer (pH 7.5) containing 0.02% $C_{12}E_9$ and 8 μg of phosphatidylcholine/mL was incubated with 10 μM NEM for 30 min at 23 °C in the presence of 2.5 mM ATP. The enzyme was then separated from NEM and ATP by gel filtration on a 15-mL Sephadex G-50 column equilibrated in the above buffer (pH 7.0) and run at 0.6 mL/min. The fractions were assayed for ATPase activity as described above, the peak fractions were pooled, and the pH was adjusted to 7.5 with NaOH.

The prelabeled enzyme (which retained 80–90% of the original ATPase activity) was then split into three 0.4-mL fractions, the first containing no additions, the second 2.5 mM AMP, and the third 2.5 mM ATP (as shown below, ATP but not AMP protected activity against inhibition by NEM and NBD-Cl). To each fraction was added 2.5 μM [^{3}H]NEM (40 Ci/mmol), and the reaction was allowed to proceed for 10 min at 23 °C. The reaction was stopped by addition of 100 μM unlabeled NEM followed by 6% trichloroacetic acid and 20 μg of cytochrome *c* (to act as a carrier protein). The samples were incubated on ice for 30 min followed by sedimentation for 5 min at 10000*g* in a Beckman Model B microfuge. The supernatants were carefully removed, and the surface of the pellets was washed with 0.2 mL of 0.15 M Tris-HCl (pH 7.5). The pellets were dissolved in 50 μL of Laemmli sample buffer containing 30% glycerol, 6% SDS, 0.19 M Tris (pH 6.8), and 0.04 mg of bromphenol blue/mL and run on an SDS–polyacrylamide gel (8%) as described below.

Prelabeling of the enzyme with nonradioactive NBD-Cl was carried out as described for NEM except that the reaction was allowed to proceed for 10 min at 23 °C. Reaction of the prelabeled enzyme with [^{14}C]NBD-Cl (2.5 μM, 109 mCi/mmol) was carried out as described for [^{3}H]NEM except that the reaction was quenched by addition of 100 μM nonradioactive NBD-Cl prior to TCA precipitation and the samples were dissolved in 50 μL of acid gel sample buffer containing 100 mM potassium phosphate (pH 4.0), 250 mM sucrose, and 70 mM cetylpyridinium chloride and run on a low-pH polyacrylamide gel as described below. Electrophoresis of [^{14}C]NBD-Cl-labeled samples was carried out at a low pH in order to reduce loss of label through exchange at high pH (Cantley & Hammes, 1975).

Gel Electrophoresis and Autoradiography. SDS–polyacrylamide gel electrophoresis was carried out using either 8% or 12% acrylamide slab gels by the method of Laemmli (1970). Acid gel electrophoresis was performed using cetylpyridinium chloride on 8% acrylamide gels as described by Amory et al. (1980). Molecular weight markers included β-galactosidase (116 000) (Sigma) and, from Pharmacia, phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400). Following electrophoresis of ^{3}H- or ^{14}C-labeled samples, gels were washed for 15 min in 30% methanol and 7.5% acetic acid, impregnated with Enhance (New England Nuclear), dried under vacuum, and exposed to Kodak XAR-5 film for 2–5 days at −70 °C using an intensifier screen. Autoradiography of gels containing ^{125}I-labeled samples was carried out without treatment of gels with Enhance. Silver staining of gels containing unlabeled samples was performed as previously described (Oakley et al., 1980).

Results and Discussion

The coated vesicle (H^+)-ATPase was partially purified by dissociation of the clathrin coat, solubilization with $C_{12}E_9$ (in the presence of phospholipid), and density gradient sedimentation on 15–30% glycerol gradients (see Materials and Methods). The partially purified enzyme had a specific activity of 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C, corresponding to a 100-fold purification relative to intact coated vesicles (the recovery of ATPase activity was 15–20%).[2] As shown in Figure 1, this preparation could be reconstituted into phospholipid vesicles to give ATP-dependent proton transport. Like the pump in intact vesicles (Stone et al., 1983; Forgac & Cantley, 1984), proton transport in reconstituted vesicles is sensitive to NEM, NBD-Cl, and the proton ionophore FCCP but is resistant to the mitochondrial (H^+)-ATPase inhibitor oligomycin. As seen in Figure 2A, the principal polypeptides present in the partially purified enzyme have molecular weights of 100 000, 73 000, 58 000, 40 000, 38 000, 34 000, 33 000, 19 000, and 17 000.[3] This is very similar to the protein pattern reported for the coated vesicle pump by Xie and Stone (1986), except for the presence in our preparation of a 19 000-dalton polypeptide. In addition, they estimate the largest protein in their preparation to have a molecular weight of 116 000.

To determine which of these polypeptides forms part of the (H^+)-ATPase complex, we have employed a series of monoclonal antibodies which recognize the coated vesicle pump. These antibodies were raised in mice immunized with intact vesicles from which the clathrin coat had been dissociated. Following removal of the spleen and fusion with a myeloma cell line, positive clones were selected for their ability to recognize the purified (H^+)-ATPase by ELISA and to precipitate the native, detergent-solubilized (H^+)-ATPase in an

[2] The specific activity obtained for the partially purified (H^+)-ATPase is comparable to the maximum specific activity observed for other purified (H^+)-ATPases, including the mitochondrial F_1 ATPases [3–4 μmol of ATP min^{-1} (mg of protein)$^{-1}$ (Cross et al., 1982)] and the plant tonoplast (H^+)-ATPase [2–3 μmol of ATP min^{-1} (mg of protein)$^{-1}$ (Mandala & Taiz, 1986)]. Thus, the subunits of the coated vesicle (H^+)-ATPase would be expected to correspond to the major polypeptides in this preparation.

[3] Although both the 38 000- and 19 000-dalton polypeptides are not heavily stained by the silver staining procedure, these proteins can be readily identified in the ^{125}I-Bolton–Hunter-labeled enzyme (Figure 2A).

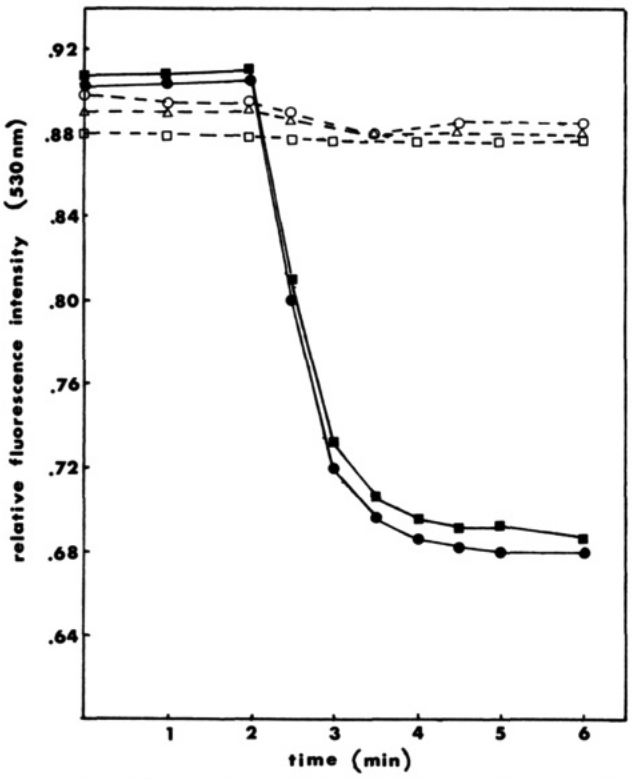

FIGURE 1: ATP-dependent acidification of reconstituted vesicles containing the partially purified coated vesicle (H^+)-ATPase. Following purification and reconstitution of the coated vesicle (H^+)-ATPase as described under Materials and Methods, acidification was measured by accumulation of acridine orange as reflected in the decrease of fluorescence intensity at 530 nm (excitation at 490 nm). Reconstituted vesicles (2 μg of protein/mL) were equilibrated in solubilization buffer containing 2 μM acridine orange followed by addition (at 2 min) of 1 mM ATP and 2 mM $MgSO_4$. ATP-dependent acidification was measured either in the absence of inhibitors (●) or in the presence of 10 μg of oligomycin/mL (■), 5 μg of FCCP/mL (□), or following incubation of the vesicles for 10 min at 23 °C with 20 μM NEM (○) or 20 μM NBD-Cl (△).

Table I: Immunoprecipitation of Coated Vesicle (H^+)-ATPase Activity[a]

culture supernatant added	protein A–Sepharose bound ATPase act. (nmol of ATP/h)	
	−(H^+)-ATPase	+(H^+)-ATPase
buffer	0.38	0.39
2C-9H (control)	0.38	0.41
1C-11G	0.33	12.20
1A-7A	0.38	12.50
2B-8B	0.41	11.35

[a] Partially purified (H^+)-ATPase from coated vesicles was incubated with the indicated monoclonal antibodies and protein A–Sepharose followed by measurement of ATPase activity bound to the Sepharose particles as described under Materials and Methods. For the column labeled "−(H^+)-ATPase", the enzyme was replaced with buffer.

active state using protein A–Sepharose (see Materials and Methods).

Table I shows the ATPase activity bound to protein A–Sepharose following incubation of the purified (H^+)-ATPase in the absence of antibody or in the presence of control antibody or each of the three positive monoclonal antibodies. As can be seen, all three positive monoclonals precipitate 25–35 times as much ATPase activity as observed in the control case. In addition, we have observed that greater than 90% of the immunoprecipitated ATPase activity is sensitive to NEM or NBD-Cl. Thus, the positive monoclonal antibodies recognize the native, detergent-solubilized (H^+)-ATPase and are capable of precipitating this enzyme in an active form. To determine

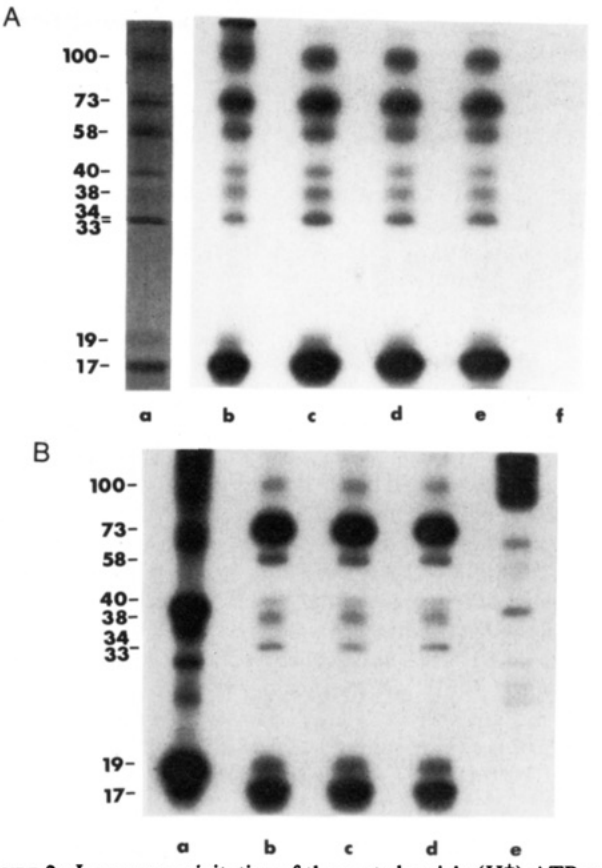

FIGURE 2: Immunoprecipitation of the coated vesicle (H^+)-ATPase using monoclonal antibodies. (A) The partially purified (H^+)-ATPase was labeled with ^{125}I-Bolton–Hunter reagent, and immunoprecipitations were carried out by using either positive or control monoclonal antibodies plus protein A–Sepharose as described under Materials and Methods; lane a, silver-stained 12% acrylamide Laemmli gel of the partially purified (H^+)-ATPase (0.2 μg of protein); lanes b–f, autoradiogram of ^{125}I-labeled (H^+)-ATPase either before immunoprecipitation (lane b) or after immunoprecipitation with the monoclonal antibodies 1C/11G (lane c), 1A/7A (lane d), 2B/8B (lane e), or 2C/9H (lane f). Each immunoprecipitation mixture contained 30 000 cpm of ^{125}I-labeled protein. (B) Vesicles were stripped of their clathrin coat and the membrane proteins solubilized with $C_{12}E_9$ and labeled with ^{125}I-Bolton–Hunter reagent as described under Materials and Methods. Shown is an autoradiogram of ^{125}I-labeled proteins either before immunoprecipitation (lane a) or after immunoprecipitation with 1C/11G (lane b), 1A/7A (lane c), 2B/8B (lane d), or 2C/9H (lane e). Each immunoprecipitation contained 300 000 cpm of ^{125}I-labeled protein. The numbers shown correspond to the polypeptide molecular weights assigned by comparison with molecular weight standards as described under Materials and Methods.

which polypeptides are precipitated by this group of monoclonal antibodies, the purified (H^+)-ATPase was labeled with ^{125}I-Bolton–Hunter reagent and the labeled enzyme incubated with the positive or control antibodies and protein A–Sepharose. As seen in Figure 2A, all the three positive monoclonals (but not the control antibody) precipitated the same set of polypeptides of molecular weight 100 000, 73 000, 58 000, 40 000, 38 000, 34 000, 33 000, 19 000, and 17 000. Although not resolvable by autoradiography, we have observed by silver staining of affinity-purified enzyme that both the 34 000- and 33 000-dalton polypeptides are immunoprecipitated by the positive monoclonals (data not shown). The monoclonals were also tested for their ability to recognize the SDS-denatured polypeptides by Western blot (see Materials and Methods). The absence of a positive reaction indicates that these antibodies are specific for the native form of the enzyme. Because the antibodies do not recognize the SDS-denatured polypeptides, it has not been possible to determine which of the

subunits each antibody is directed against.[4]

To further test the specificity of the immunoprecipitation, stripped vesicles were solubilized with detergent, labeled with ^{125}I-Bolton–Hunter reagent, and incubated with the control or positive monoclonals and protein A–Sepharose as described above. Despite the complex mixture of proteins present in the detergent-solubilized membranes, the positive monoclonals precipitated the same set of polypeptides as observed with the purified enzyme (Figure 2B).[5] These results thus indicate that this set of polypeptides forms a single macromolecular complex and suggest that they correspond to subunits of the coated vesicle (H^+)-ATPase.

To identify which of the polypeptides present in the (H^+)-ATPase complex is associated with ATP hydrolysis, two ATP-protectable inhibitors of the coated vesicle proton pump, NEM and NBD-Cl, were employed. We previously demonstrated that NBD-Cl is a potent inhibitor of both proton transport and ATPase activity in intact coated vesicles and that this inhibition is prevented in the presence of ATP (Forgac & Cantley, 1984). NBD-Cl has also been shown to inhibit the ATP-dependent proton pumps present in chromaffin granules (Dean et al., 1984), Golgi-derived vesicles (Glickman et al., 1983), and the vacuolar membranes of *Neurospora* (Bowman, 1983), yeast (Uchida et al., 1985), and plants (Wang & Sze, 1985). NEM sensitivity has been demonstrated for all of the proton pumps which are inhibited by NBD-Cl as well as the endosomal and lysosomal proton pumps (Galloway et al., 1983; Moriyama et al., 1985). As demonstrated in Figure 3, the purified coated vesicle (H^+)-ATPase is also sensitive to low concentrations of NEM and NBD-Cl, and this inhibition is prevented by ATP but not AMP. These results suggest that both NEM and NBD-Cl inhibit activity of the coated vesicle pump by reaction at an ATP binding site on the enzyme, possibly the catalytic site.

Labeling of the coated vesicle (H^+)-ATPase was carried out by using [^{3}H]NEM and [^{14}C]NBD-Cl in the absence or presence of nucleotides. One difficulty with both NEM and NBD-Cl is reaction at sites unrelated to their effects on activity. To increase the specificity of the labeling reaction by reducing the number of such nonspecific sites, we employed the following procedures. The (H^+)-ATPase was first reacted with nonradioactive NEM or NBD-Cl in the presence of ATP. In this way, the sites responsible for inhibition of activity are protected. Following removal of the ATP and nonradioactive reagents by gel filtration, the prelabeled enzyme was then reacted with [^{3}H]NEM or [^{14}C]NBD-Cl in the absence of nucleotides or in the presence of AMP or ATP. The reactive sites responsible for inhibition of ATPase activity should therefore be labeled by [^{3}H]NEM or [^{14}C]NBD-Cl, and this labeling should be prevented by ATP but not AMP. In addition, a lower concentration of radioactive reagent was used in the labeling experiments (relative to the prelabeling conditions), since this lower concentration was sufficient to inhibit ATPase activity in an ATP-protectable manner (Figure 3) while minimizing reaction at nonspecific sites.

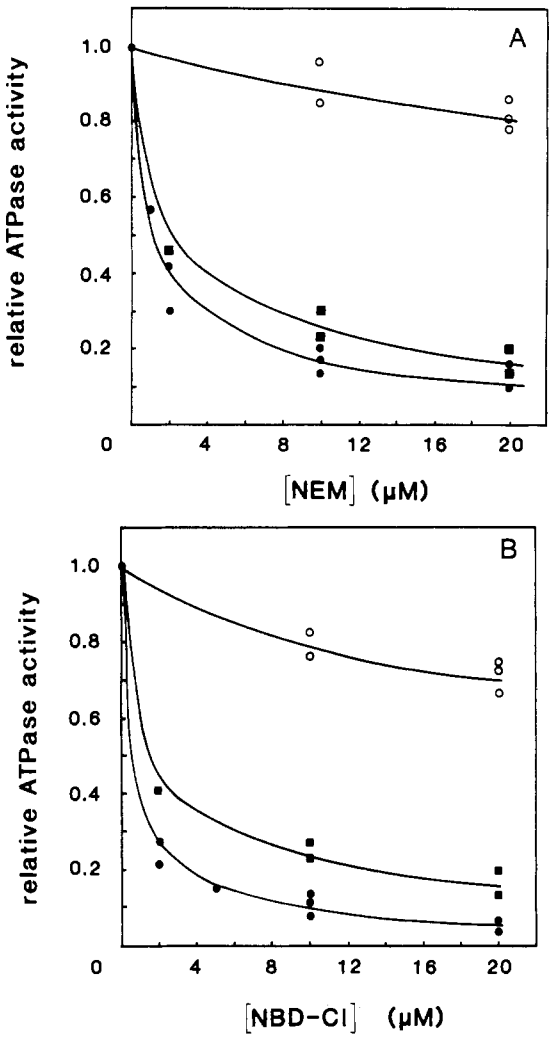

FIGURE 3: NEM and NBD-Cl inhibition of (H^+)-ATPase activity. The (H^+)-ATPase from coated vesicles was purified as described under Materials and Methods to give a preparation of specific activity 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C. The partially purified enzyme (3 μg of protein/mL) was then treated with the indicated concentrations of NEM (A) or NBD-Cl (B) in solubilization buffer (pH 7.5) containing 0.02% $C_{12}E_9$ and 8 μg of phosphatidylcholine/mL for 10 min at 23 °C either in the absence of nucleotides (●) or in the presence of 2.5 mM AMP (■) or 2.5 mM ATP (○). ATPase activity was then measured by the spectrophotometric assay described under Materials and Methods.

Figure 4 shows the densitometer scans of the autoradiograms obtained following labeling of the coated vesicle (H^+)-ATPase with [^{3}H]NEM and [^{14}C]NBD-Cl as described above, while the quantitation of labeling is given in Table II. As can be seen, although many of the polypeptides present in the purified enzyme are labeled to some extent by both [^{3}H]NEM and [^{14}C]NBD-Cl, the principal polypeptide labeled in both cases has a molecular weight of 73 000. By densitometry, it is estimated that this 73 000-dalton protein contains 38.5% of the [^{3}H]NEM and 47.6% of the [^{14}C]NBD-Cl incorporated into the complex (Table II). The observation that multiple polypeptides in the (H^+)-ATPase complex were labeled by [^{3}H]NEM and [^{14}C]NBD-Cl indicates that prelabeling of the enzyme with the nonradioactive reagents in the presence of ATP did not saturate all of the nonspecific sites on the protein.

[4] We have observed quantitative differences in the ability of the three positive monoclonal antibodies to immunoprecipitate the (H^+)-ATPase in intact vesicles (data not shown), suggesting that differences exist in the accessibility of the antibodies to their respective antigenic sites when the pump is surrounded by the remaining coated vesicle proteins. This observation indicates that the three positive monoclonal antibodies are different from one another but does not exclude the possibility that they all recognize the same subunit.

[5] When the (H^+)-ATPase is purified from crude mixtures by immunoaffinity chromatography using the monoclonal antibody 2B-8B covalently linked to Sepharose, we have found that the relative subunit intensities obtained by silver staining are the same as those observed for the (H^+)-ATPase purified by the method described. Thus, the differences in relative subunit intensities observed between Figure 2A and Figure 2B are most likely due to differences in labeling of the enzyme by ^{125}I-Bolton–Hunter reagent in the two environments rather than to significant differences in protein composition.

Table II: Quantitation of [^{3}H]NEM and [^{14}C]NBD-Cl Labeling of Coated Vesicle (H^+)-ATPase[a]

	relative label incorporated							
	[^{3}H]NEM			[^{14}C]NBD-Cl			ATP/AMP ratio[b]	
polypeptide M_r	control	+AMP	+ATP	control	+AMP	+ATP	NEM	NBD-Cl
120 000				0.149	0.143	0.136		0.95
100 000	0.159	0.147	0.132	0.114	0.097	0.102	0.90	1.05
73 000	0.385	0.363	0.150	0.476	0.418	0.190	0.41	0.45
58 000	0.323	0.304	0.306	0.261	0.253	0.263	1.01	1.04
40 000	0.133	0.122	0.114				0.93	

[a] The fraction of [^{3}H]NEM or [^{14}C]NBD-Cl incorporated into each of the polypeptides of the coated vesicle (H^+)-ATPase in the absence of nucleotides was determined from the densitometer scans shown in Figure 5, and the values are reported in the column labeled "control". Only bands containing ≥5% of the total label incorporated are listed. Although not resolvable on 8% polyacrylamide gels, we have observed that neither the 19 000- nor the 17 000-dalton polypeptides are labeled by either reagent to >5% (data not shown). The values reported under "+AMP" and "+ATP" represent the incorporation observed in the presence of the corresponding nucleotide relative to the control values. [b] Values represent the ratio of label incorporated in the presence of ATP divided by label incorporated in the presence of AMP.

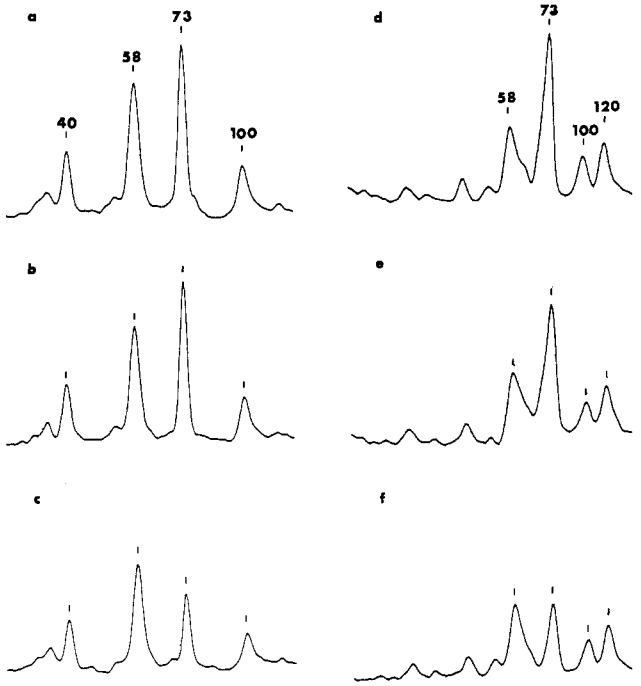

FIGURE 4: [^{3}H]NEM and [^{14}C]NBD-Cl labeling of the coated vesicle (H^+)-ATPase. The partially purified (H^+)-ATPase (14 μg of protein) was first reacted with nonradioactive NEM (panels a–c) or NBD-Cl (panels d–f) at 10 μM in the presence of 2.5 mM ATP. After removal of the unreacted reagents and ATP by gel filtration, the prelabeled enzyme was reacted with 2.5 μM [^{3}H]NEM (panels a–c) or 2.5 μM [^{14}C]NBD-Cl (panels d–f) for 10 min at 23 °C either in the absence of nucleotides (panels a and d) or in the presence of 2.5 mM AMP (panels b and e) or 2.5 mM ATP (panels c and f). The reactions were quenched by addition of 100 μM nonradioactive reagent, and the protein was precipitated by addition of 6% trichloroacetic acid. After the precipitates were washed, they were solubilized in sample buffer and subjected to polyacrylamide gel electrophoresis on 8% acrylamide gels using the method of Laemmli (1970) (panels a–c) or Oakley et al. (1980) (panels d–f). [^{14}C]NBD-Cl-labeled samples were subjected to electrophoresis at low pH to avoid loss of label due to exchange at high pH (Cantley & Hammes, 1975). Autoradiography was carried out as described under Materials and Methods, and autoradiograms were scanned by using an LKB Model 2202 laser densitometer.

Saturation of these nonspecific sites is likely to occur only under conditions which also result in reaction of the ATP-protectable sites.

In addition to being the principal polypeptide labeled by both [^{3}H]NEM and [^{14}C]NBD-Cl, the 73 000-dalton protein is the only one to show significant protection by ATP. ATP reduces [^{3}H]NEM labeling of the 73 000-dalton polypeptide by 59% and [^{14}C]NBD-Cl labeling by 55%. In addition, as shown in Table II, this reduction in labeling is not observed in the presence of AMP. Although the ATP protection observed in the labeling experiments is less than that observed for activity (Figure 3), this may be accounted for by reaction of the 73 000-dalton protein with [^{3}H]NEM and [^{14}C]NBD-Cl at sites other than the ATP binding site. Thus, the 73 000-dalton polypeptide has the properties expected for the subunit whose reaction with NEM and NBD-Cl is responsible for inhibition of the coated vesicle (H^+)-ATPase by these reagents. Moreover, the nucleotide dependence of this reaction suggests that the 73 000-dalton protein possesses an ATP binding site, possibly the catalytic site.

It is of interest to compare these results with labeling studies carried out on closely related proton pumps present in other intracellular membranes of eukaryotic cells. The plant tonoplast (H^+)-ATPase appears to contain three polypeptides of molecular weights 72 000, 60 000, and 17 000 (Randall & Sze, 1986; Mandala & Taiz, 1986), and, as with the coated vesicle pump, the 72 000-dalton protein is labeled with [^{14}C]NBD-Cl in an ATP-protectable manner (Mandala & Taiz, 1986). The vacuolar (H^+)-ATPase from *Neurospora* appears to have a subunit composition identical with the plant tonoplast enzyme, and, as with the coated vesicle (H^+)-ATPase, a 70 000-dalton polypeptide is labeled by both [^{14}C]NEM and [^{14}C]NBD-Cl in an ATP-protectable manner (Bowman et al., 1986). Thus, a polypeptide of molecular weight 70 000–73 000 is implicated as an ATP binding subunit for all three (H^+)-ATPases, suggesting that these enzymes may be derived from a common evolutionary ancestor.

By contrast, the chromaffin granule (H^+)-ATPase has been reported to contain four polypeptides of molecular weight 115 000, 72 000, 57 000, and 39 000 (Cidon & Nelson, 1986). Moreover, the 115 000-dalton protein was postulated to serve as the ATP-hydrolytic site on the basis of its reaction with [^{14}C]NEM and [^{14}C]DCCD, although no ATP protection of labeling of this polypeptide was reported. The 100 000-dalton polypeptide of the coated vesicle pump was labeled by both [^{3}H]NEM and [^{14}C]NBD-Cl, but no ATP protection of this labeling was observed. Similarly, a 120 000-dalton polypeptide was labeled by [^{14}C]NBD-Cl (Figure 4), but, again, this labeling was not inhibited by ATP. In addition, this 120 000-dalton protein was not labeled by [^{3}H]NEM or immunoprecipitated by using the positive monoclonal antibodies. It should be noted, however, that our results do not exclude the possible involvement of other subunits of the (H^+)-ATPase complex in ATP hydrolysis. Rather, they indicate that the 73 000-dalton polypeptide is at least one of the subunits involved in ATP binding and catalysis. The function of the remaining subunits in this complex is currently being studied.

ACKNOWLEDGMENTS

We thank Dr. Gilbert Chin, Howard Hughes Medical In-

stitute, Columbia University, for many helpful discussions.
Registry No. ATPase, 9000-83-3; ATP, 56-65-5.

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lac Permease of *Escherichia coli*: Arginine-302 as a Component of the Postulated Proton Relay

Donald R. Menick, Nancy Carrasco,[‡] Lisa Antes,[§] Lekha Patel, and H. Ronald Kaback*
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110
Received April 1, 1987; Revised Manuscript Received June 8, 1987

ABSTRACT: The *lac* permease of *Escherichia coli* was modified by site-directed mutagenesis such that Arg-302 in putative helix IX was replaced with Leu. In addition, Ser-300 (helix IX) was replaced with Ala, and Lys-319 in putative helix X was replaced with Leu. Permease with Leu at position 302 manifests properties that are similar to those of permease with Arg in place of His-322 [Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483]. Thus, permease with Leu-302 is markedly defective in active lactose transport, efflux, exchange, and counterflow but catalyzes downhill influx of lactose at high substrate concentrations without H^+ translocation. In contrast, permease molecules with Ala at position 300 or Leu at position 319 catalyze lactose/H^+ symport in a manner indistinguishable from that of wild-type permease. By molecular modeling, Arg-302 may be positioned in helix IX so that it faces the postulated His-322/Glu-325 ion pair in helix X. In this manner, the guanidino group in Arg-302 may interact with the imidazole of His-322 and thereby play a role in the H^+ relay suggested to be involved in lactose/H^+ symport [Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486].

lac permease of *Escherichia coli* is a hydrophobic transmembrane protein encoded by the *lac Y* gene that catalyzes symport of β-galactosides with H^+ [cf. Kaback (1986a,b) and Wright et al. (1986) for reviews]. Therefore, when a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] is generated across the cytoplasmic membrane (interior negative and alkaline), the permease utilizes free energy released from downhill trans-

[‡] Present address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, New York, NY 10461.
[§] Present address: Rutgers Medical School, Piscataway, NJ 08854.

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; Dns^6-Gal, 6-(*N*-dansylamino)hexyl 1-thio-β-D-galactopyranoside; ss, single stranded; cc, closed circular; RF, replicative form; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RSO, right side out; PMS, phenazine methosulfate; Mab, monoclonal antibody.

0006-2960/87/0426-6638$01.50/0