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Structural Characterization of the ATP-Hydrolyzing Portion of the Coated Vesicle Proton Pump†

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ABSTRACT: The ATP-hydrolyzing portion of the proton pump from clathrin-coated vesicles (isolated from calf brain) was solubilized with three nondenaturing detergents (cholate, octyl glucoside, and Triton X-100). The hydrodynamic properties of the solubilized (Mg^{2+})-ATPase were then determined by sedimentation analysis in H_2O and D_2O and gel filtration on Sepharose 4B. The coated vesicle (Mg^{2+})-ATPase migrated under all conditions as a single peak of activity. In cholate, the sedimentation coefficient ($s_{20,w}$), Stokes radius (a), and partial specific volume ($\bar{v}_c$) were 8.25 (±0.20) S, 68 (±2) Å, and 0.71 (±0.03) cm^3/g, respectively. In octyl glucoside and Triton X-100 these values were respectively 7.90 (±0.20) and 7.45 (±0.20) S, 68 (±3) and 101 (±5) Å, and 0.74 (±0.03) and 0.75 (±0.03) cm^3/g. Application of the Svedberg equation to these data gave a molecular weight for the protein–detergent complex of 217 000 ± 21 000 (cholate), 234 000 ± 26 000 (octyl glucoside), and 337 000 ± 40 000 (Triton X-100). Assuming the protein binds one micelle of detergent, these values correspond to a protein molecular weight of 215 000 ± 21 000 (cholate), 226 000 ± 26 000 (octyl glucoside), and 247 000 ± 40 000 (Triton X-100). The cholate-solubilized, gradient-purified (Mg^{2+})-ATPase, when combined with a 100000g pellet fraction, could be reconstituted by dialysis into phospholipid vesicles which displayed ATP-dependent proton uptake. The sensitivity of this acidification and the (Mg^{2+})-ATPase activity of the detergent-solubilized protein to 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and *N*-ethylmaleimide (NEM) and their resistance to ouabain are consistent with this protein complex corresponding to the ATP-hydrolyzing portion of the coated vesicle proton pump.

Results obtained in a variety of systems have suggested that exposure to low pH in a prelysosomal compartment is the signal which activates ligand–receptor dissociation following receptor-mediated endocytosis (Tycko & Maxfield, 1982; Merion & Sly, 1983; Marsh et al., 1983; Harford et al., 1983). Clathrin-coated vesicles have been shown to contain an ATP-dependent proton pump which has been postulated to be responsible for this acidification (Forgac et al., 1983; Stone et al., 1983). The coated vesicle pump is similar in inhibitor sensitivity and ionic requirements to a number of other intracellular proton pumps, including those present in endosomes (Galloway et al., 1983; Yamashiro et al., 1983), lysosomes (Schneider, 1981; Harikumar & Reeves, 1983), and Golgi-derived vesicles (Glickman et al., 1983; Zhang & Schneider, 1983), but its relationship to these pumps remains uncertain. We have also demonstrated that the coated vesicle pump shares a number of important properties with the mitochondrial (H^+)-ATPase, including the absence of a phosphorylated intermediate (Forgac & Cantley, 1984), although these proteins are distinguishable on the basis of their sensitivity to oligomycin and aurovertin (Forgac et al., 1983). Reconstitution

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of the partially purified pump has been reported (Xie et al., 1984), but the structure of this pump remains uncertain. In the present paper we report structural information on the detergent-solubilized (Mg^{2+})-ATPase[1] obtained from sedimentation analysis and gel filtration and describe reconstitution of this activity by cholate dialysis.

Materials and Methods

Materials. Calf brains were obtained fresh from a local slaughterhouse. Phosphatidylcholine was prepared from fresh egg yolk by chloroform/methanol extraction as previously described (Forgac & Chin, 1981) and stored as a suspension (140 mg/mL) in water containing 5 mM 2-mercaptoethanol at −70 °C under nitrogen. Phosphatidylserine was obtained as a chloroform solution from Avanti Polar Lipids, Inc., and stored at −20 °C. Cholic acid, octyl glucoside, Triton X-100, and poly(oxyethylene) 9-lauryl ether ($C_{12}E_9$) were obtained from Sigma Chemical Co., and cholic acid was decolorized by using activated charcoal and recrystallized from 95% ethanol. Solutions of sodium cholate were prepared by neutralization with NaOH.

The following sedimentation and gel filtration markers were obtained from Sigma Chemical Co.: thyroglobulin (porcine), β-galactosidase (*Escherichia coli*), catalase (bovine liver), fumarase (porcine heart), aldolase (rabbit muscle), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (porcine heart), and cytochrome *c* (bovine heart). Pyruvate kinase (rabbit muscle), lactate dehydrogenase (bovine heart), NADH (grade III), and phosphoenolpyruvate (tricyclohexylammonium salt) were obtained from the same source and used in the ATPase assay described later.

Disodium ATP (grade II), ouabain, valinomycin, *N*-ethylmaleimide, and all substrates for marker enzymes were purchased from Sigma. 7-Chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and acridine orange were purchased from Eastman Kodak and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was purchased from Aldrich Chemical Co.

Buffers. Buffers had the following compositions. Buffer A (used in preparation of coated vesicles) contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 34 mM NaCl, 0.5 mM $MgCl_2$, 1.0 mM EGTA, 0.02% sodium azide, and 0.2 mM dithiothreitol. Stripping buffer (used in removal of peripheral proteins from the coated vesicles) contained 5 mM Tris (pH 8.5), 0.5 mM EDTA, and 150 mM sucrose. Solubilization buffer [used in solubilization of the (Mg^{2+})-ATPase and in all other experiments described] contained 50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 100 mM sucrose, and 0.2 mM EGTA.

Assays. ATPase activity was measured by a continuous spectrophotometric assay using a Kontron Model 820 spectrophotometer as previously described (Cantley & Josephson, 1976). Assays were carried out in solubilization buffer containing 1.0 mM ATP, 2.0 mM $MgSO_4$, 1.5 mM phosphoenolpyruvate, 0.25 mg of NADH/mL, 20 μg of pyruvate kinase/mL, and 20 μg of lactate dehydrogenase/mL at 37 °C. When detergent-solubilized preparations were assayed, 0.02% $C_{12}E_9$, 8 μg of phosphatidylcholine/mL, and 4 μg of phosphatidylserine/mL were added to the assay mixture to prevent the proteins from aggregating. Unless otherwise indicated, assays were also carried out in the presence of 100 μM ouabain to inhibit any (Na^+,K^+)-ATPase activity present.

Proton transport was measured by acridine orange fluorescence quenching by using a Perkin-Elmer LS-5 spectrofluorometer as previously described (Lee & Forte, 1978). Assays were carried out in solubilization buffer in a total volume of 0.5 mL containing 2 μM acridine orange at 23 °C. After equilibration of the vesicles with acridine orange for 10–15 min at room temperature, 1.0 mM ATP and 2.0 mM $MgSO_4$ were added simultaneously, and the fluorescence intensity at 530 nm (excitation at 490 nm) was monitored. The degree of fluorescence quenching was linearly related to the amount of coated vesicle protein added to the assay up to a 40% quenching provided that a correction was made for the 2–4% increase in fluorescence observed on addition of MgATP to a solution of acridine orange alone.

Marker enzymes were assayed spectrophotometrically by using a Kontron Model 820 recording spectrophotometer in solubilization buffer at 37 °C as previously described (Clarke, 1975). Cytochrome *c* was monitored by the absorbance at 550 nm while thyroglobulin was monitored at 280 nm. The protein concentration was determined by the method of Lowry et al. (1951) in the presence of 1.0% sodium dodecyl sulfate.

Preparation and Stripping of Clathrin-Coated Vesicles. Clathrin-coated vesicles were prepared from calf brain by differential centrifugation, D_2O–Ficoll gradient sedimentation, and Sephacryl S-1000 chromatography as previously described (Wiedenmann & Mimms, 1983). Vesicles were stored in buffer A at 4 °C and used within 2 weeks of preparation or were quick-frozen in dry ice–ethanol and stored at −70 °C until used.

Vesicles were stripped of their clathrin coats as previously described (Forgac & Cantley, 1984). Approximately 70–80% of both proton transport activity and (Mg^{2+})-ATPase activity were retained by the vesicles while 90–95% of the total protein (largely the M_r 180 000 clathrin heavy chain) remained in the supernatant. Thus, an approximately 10-fold purification of the proton pump was achieved in this step. Stripped vesicles were stored at 4 °C and used within 1 week of their preparation.

Detergent Solubilization of the Coated Vesicle Proton Pump. Stripped vesicles were solubilized with sodium cholate as follows. A stock solution containing 5.0% cholate and 1.0 mg of phosphatidylserine/mL in solubilization buffer was prepared by first drying a chloroform solution of phosphatidylserine under nitrogen and then dissolving the lipid in solubilization buffer containing 5.0% sodium cholate (pH 7.0) by vortexing at room temperature until clear. For some experiments phosphatidylcholine (2 mg/mL) was also added to this stock solution. The detergent/phospholipid solution was diluted into solubilization buffer followed by addition of stripped vesicles such that the final concentrations were 0.5 mg of protein/mL, 1.5% sodium cholate, and 0.30 mg of phosphatidylserine/mL (in some cases 0.60 mg of phosphatidylcholine/mL was also present). This mixture was vortexed and incubated for 20 min at 23 °C followed by sedimentation at 4 °C for 1 h at 100000*g*. The supernatant was removed and used as described later.

Solubilization with octyl glucoside and Triton X-100 was carried out in an identical manner except that the concentration of detergent in the solubilization mixture was 1.0% (but with the same ratio of phospholipid to detergent as described earlier). The recovery of (Mg^{2+})-ATPase activity in the 100000*g* supernatant was in all cases 40–60% whereas the

[1] Abbreviations: (Mg^{2+})-ATPase, Mg^{2+}-dependent adenosine triphosphatase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NEM, *N*-ethylmaleimide; 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; $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

recovery of protein was 50–70%. Thus, no significant purification of the (Mg^{2+})-ATPase was obtained by this step.

Sucrose Gradient Sedimentation. Samples of detergent-solubilized vesicles (300–400 μL) were applied to 11-mL linear 5–25% sucrose gradients prepared in solubilization buffer containing either 0.65% cholate, 0.80% octyl glucoside, or 0.05% Triton X-100 (the detergent present in the gradient was the same as that used in solubilization). In all cases phosphatidylserine was present in a ratio of 1:50 (w/w) phospholipid:detergent [for some gradients phosphatidylcholine was also present at a ratio of 1:25 w/w) phospholipid:detergent]. Marker enzymes were added directly to the detergent-solubilized samples or run in parallel gradients under identical conditions. Gradients run in H_2O were centrifuged at 4 °C for 32 h at 38 000 rpm (175000*g*) in a Beckman SW 41 rotor using a Beckman L5-75B ultracentrifuge. Gradients run in D_2O were centrifuged under identical conditions except for 44 h.

After sedimentation, gradients were fractionated from the bottom by using a Buchler peristaltic pump and Gilson microfractionator to give 16–18 fractions of 0.63–0.71 mL per fraction. The fractions were assayed for (Mg^{2+})-ATPase and marker enzyme activities as described earlier. The recovery of (Mg^{2+})-ATPase activity following sedimentation varied from 60 to 80% except for gradients run in cholate in the absence of phosphatidylcholine in which case the recovery was 40%. In all cases the (Mg^{2+})-ATPase appeared as a single peak of activity. The sedimentation coefficient of this peak was determined by comparison with the sedimentation coefficients of the marker proteins. Values of the sedimentation coefficient ($s_{20,w}$) and Stokes radius (*a*) of the marker proteins employed (Sober, 1970; Clarke, 1975; Aiyer, 1983) are as follows: thyroglobulin (19.0 S; 86 Å), β-galactosidase (15.9 S; 69 Å), catalase (11.3 S; 52 Å), fumarase (9.1 S; 53 Å), aldolase (7.7 S; 45 Å), lactate dehydrogenase (7.0 S; 42 Å), malate dehydrogenase (4.3 S; 35 Å), and cytochrome *c* (1.7 S; 19 Å).

The partial specific volume ($\bar{v}_c$) of the (Mg^{2+})-ATPase in various detergents was determined from the sedimentation coefficients measured in H_2O and D_2O as described by Clarke [eq 14 (Clarke, 1975)]. Values for the densities and relative viscosities of the sucrose solutions at the average positions of migration were obtained from the *Handbook of Chemistry and Physics* (1972).

Gel Filtration. Samples of detergent-solubilized membranes (300–400 μL) were applied to a 36 × 1.6 cm Sepharose 4B column equilibrated with solubilization buffer containing either 0.65% cholate, 0.80% octyl glucoside, or 0.05% Triton X-100 plus phospholipids added in the same ratio to detergent as described earlier. Marker enzymes were included in the sample applied to the column. The column was eluted at a rate of 3.1–3.7 mL/h, and 15-min fractions were collected on a Gilson microfractionator. The fractions were assayed for (Mg^{2+})-ATPase and marker enzyme activities as described earlier. As with the sedimentation analysis, the (Mg^{2+})-ATPase activity migrated as a single peak. The Stokes radius of the peak was determined by comparison with the Stokes radii of the marker proteins (given earlier).

Calculation of Molecular Weights. The molecular weight of the detergent-solubilized complex (M_c) was calculated by using the Svedberg equation:

$$M_c = \frac{s_c 6\pi\eta a_c N}{1 - \bar{v}_c\rho}$$

where s_c is the sedimentation coefficient of the complex, η and ρ are the viscosity and density of the solution, $\bar{v}_c$ and *a* are the partial specific volume and Stokes radius of the complex, and *N* is Avagadro's number (Clarke, 1975). The molecular weight of the protein portion of the complex (Table I) was estimated by assuming each protein molecule binds to one micelle of detergent. This assumption has been shown to be valid for a large number of membrane proteins (Clarke, 1975; Aiyer, 1983). The amount of phospholipid bound was estimated from the partial specific volume of the complex [see Results and Discussion and Steele et al. (1978)]. The values of the micellar molecular weight and partial specific volume ($\bar{v}_d$) of the detergents employed (Hjelmemand & Chrambach, 1984; Steele et al., 1978) are as follows: sodium cholate (1700; 0.75 cm^3/g), octyl glucoside (8000; 0.925 cm^3/g), and Triton X-100 (90 000; 0.908 cm^3/g). The partial specific volume of octyl glucoside was calculated as described by Steele et al. (1978), from the data in Hoiland and Vikingstad (1976).

Reconstitution of the Coated Vesicle Proton Pump. The peak of (Mg^{2+})-ATPase activity from the sucrose gradient containing cholate (plus phospholipids) was reconstituted into sealed phospholipid vesicles as follows. The peak fractions from the gradient (fractions 5 and 6) were pooled and concentrated to 0.5 mL by using a Centricon 10 microconcentrator (Amicon), and 2 mM 2-mercaptoethanol was added. The pooled fractions were then combined with 50 μL of 5% cholate (containing 1 mg of phosphatidylserine/mL and 2 mg of phosphatidylcholine/mL) and 100 μL of a resuspended 100000*g* pellet fraction (0.20 mg of protein/mL) obtained after centrifugation of the cholate-solubilized membranes. As previously reported (Xie et al., 1984), addition of this 100000*g* pellet was necessary to obtain reconstitution of proton pumping. This mixture was incubated for 1 h at 23 °C, and then 100 μL of 10% cholate (pH 7.0), 7 mg of phosphatidylcholine/mL, 4 mg of cholesterol/mL, 150 mM NaCl, and 2 mM 2-mercaptoethanol was added with vortexing. The mixture was incubated an additional 20 min at 23 °C and then transferred to 6.4 mm diameter Spectrapor 2 dialysis tubing (Amicon) with a molecular weight cutoff of 12 000–14 000. The sample was dialyzed for 4 days at 4 °C against four changes of 500 volumes of solubilization buffer. As a control, the remaining gradient fractions were pooled, concentrated, and carried through an identical procedure. Using [^{3}H]cholic acid (25 Ci/mmol; New England Nuclear), we observed that this procedure resulted in the removal of 99.99% of the starting cholate. Attempts to reconstitute the octyl glucoside and Triton X-100 solubilized enzymes proved unsuccessful, possibly due to incorrect orientation of the reconstituted protein or to incomplete removal of the detergent during dialysis. Acidification in the reconstituted vesicles was measured as described earlier.

Results and Discussion

The purpose of this study was to obtain structural information on the proton pump present in clathrin-coated vesicles (Forgac et al., 1983; Stone et al., 1983). Previous results have suggested that, at least in clathrin-coated vesicles isolated from calf brain, the proton pump corresponds to the principal ATPase activity. Thus, parallel inhibition of proton transport and ATPase activity has been observed for both NBD-Cl (Forgac & Cantley, 1984) and NEM (Stone et al., 1983), and ionophores such as FCCP and valinomycin cause significant stimulation of ATP hydrolysis (Forgac et al., 1983). These results are consistent with the proton pump corresponding to a significant fraction of the total ATPase activity and suggest that it is possible to use ATP hydrolysis to monitor the behavior of the proton pump following detergent solubilization. Using

a combination of sedimentation analysis in H_2O and D_2O and gel filtration as described by Clarke (1975), we have determined the hydrodynamic properties of the detergent-solubilized (Mg^{2+})-ATPase from clathrin-coated vesicles. To test the validity of this approach, we have also reconstituted this (Mg^{2+})-ATPase activity into phospholipid vesicles (as will be discussed).

Three nondenaturing detergents were employed to solubilize the (Mg^{2+})-ATPase from clathrin-coated vesicles:[2] sodium cholate, octyl glucoside, and Triton X-100. As previously reported (Xie et al., 1984), phosphatidylserine was found to stabilize ATPase activity. Thus, 40–60% of the (Mg^{2+})-ATPase activity could be recovered in the 100000*g* supernatant by using any of the three detergents provided that phosphatidylserine was present at a ratio to detergent of 1:50 (w/w). Cholate and octyl glucoside were selected for their high critical micelle concentration (see Materials and Methods), thus facilitating their removal by dialysis during reconstitution. Triton X-100 was used because of the data available on the hydrodynamic properties of other membrane protein–Triton complexes (Clarke, 1975; Aiyer, 1983). Detergents were employed throughout the analysis at concentrations above their critical micellar concentrations in order to maintain the proteins in a soluble state. Phosphatidylserine was also present at a constant ratio to detergent (1:50 w/w) to preserve (Mg^{2+})-ATPase activity.

Figure 1a shows the sedimentation profile for the cholate-solubilized enzyme in H_2O. The (Mg^{2+})-ATPase migrated as a single peak of activity, an observation that applied in all detergents throughout the sedimentation and gel filtration analysis. By comparison with the sedimentation coefficients of the marker enzymes, a sedimentation coefficient for the detergent-solubilized complex of 7.50 (±0.15) S was determined. Figure 1b shows the sedimentation profile for the cholate-solubilized enzyme in D_2O. The shift to lighter density relative to the marker enzymes indicates the higher partial specific volume of the detergent-solubilized complex. By use of the sedimentation behavior in H_2O and D_2O and the equation described by Clarke (1975), a partial specific volume for the complex of 0.76 (±0.02) cm^3/g was calculated. These data were obtained for the cholate-solubilized enzyme in the presence of both phosphatidylserine and phosphatidylcholine (at ratios to detergent of 1:50 and 1:25, respectively). If only cholate and phosphatidylserine were present during solubilization and sedimentation, a sedimentation coefficient of 8.25 (±0.20) S and a partial specific volume of 0.71 (±0.03) cm^3/g were observed. These results indicate that phosphatidylcholine [$\bar{v}$ = 0.98 cm^3/g (Tanford & Reynolds, 1976)] does form part of this complex, thus increasing its partial specific volume.

From gel filtration of the cholate-solubilized (Mg^{2+})-ATPase on Sepharose 4B, a Stokes radius of 70 (±2) Å was determined for the complex. From the sedimentation coefficient, the partial specific volume, and the Stokes radius, a molecular weight for the detergent-solubilized complex of 247000 (±19000) was calculated by using the Svedberg equation as described under Materials and Methods. The data obtained in the absence of phosphatidylcholine gave a complex molecular weight of 217000 (±21000).

To calculate the contribution of the protein to the molecular weight of the complex, it is necessary to know the amount of detergent and phospholipid bound. Previous studies have demonstrated that, for those membrane proteins studied, each protein molecule binds one micelle of detergent (Clarke, 1975; Aiyer, 1983). Because of the small size of the cholate micelle (M_r 1700), the contribution of the detergent to the size of the complex will be small. Although the contribution of the phosphatidylserine to the complex cannot be determined precisely, the observation that the partial specific volume of the complex (0.71 cm^3/g) is much closer to the value obtained for most soluble proteins [0.70–0.75 cm^3/g (Clarke, 1975)] than to that of phosphatidylserine [0.93 cm^3/g (Steele et al., 1978)] implies that phosphatidylserine does not make a significant contribution to the molecular weight of the complex.

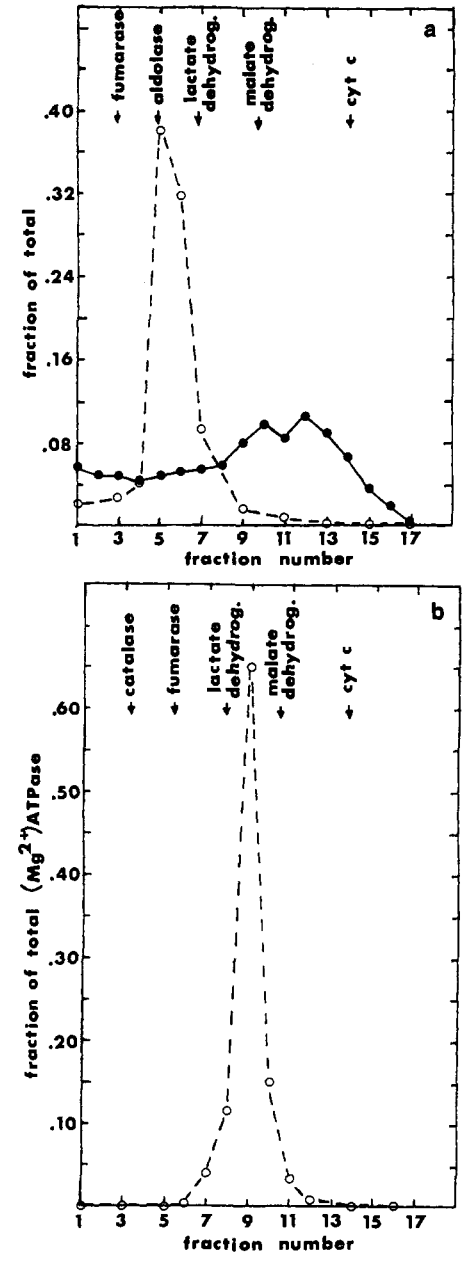

FIGURE 1: Sucrose density gradient sedimentation of cholate-solubilized coated vesicle (Mg^{2+})-ATPase in H_2O and D_2O. Stripped vesicles (0.50 mg of protein/mL) were solubilized with 1.5% sodium cholate (0.6 mg of phosphatidylcholine/mL, 0.3 mg of phosphatidylserine/mL) and centrifuged for 1 h at 100000*g*. Marker enzymes were added, and the supernatant (300 μL) was applied to an 11-mL 5–25% linear sucrose gradient containing 0.65% cholate (plus phospholipids) in solubilization buffer prepared in either H_2O (a) or D_2O (b). Gradients were centrifuged at 4 °C in an SW 41 rotor at 38000 rpm for either 32 (H_2O) or 44 h (D_2O) and fractionated (0.66 mL/fraction), and the fractions were assayed for (○) (Mg^{2+})-ATPase activity and (●) protein or marker enzyme activities (Materials and Methods). ATPase activity and protein are given as a fraction of the total recovered.

[2] Vesicles from which the clathrin coat had been stripped were used as a starting material because of their 10-fold higher specific activity with respect to both proton transport and ATP hydrolysis.

Table I: Hydrodynamic Properties of the Coated Vesicle Proton Pump[a]

detergent	±PC	$s_{20,w}$ ($\times 10^{13}$ s)	Stokes radius (Å)	$\bar{\nu}_c$ (cm^3/g)	$M_{complex}$ ($\times 10^{-3}$)	$M_{protein}$[b] ($\times 10^{-3}$)
sodium cholate	−	8.25 (±0.20)	68 (±2)	0.71 (±0.03)	217 (±21)	215 (±21)
	+	7.50 (±0.15)	70 (±2)	0.76 (±0.02)	247 (±19)	200 (±19)
octyl glucoside	−	7.90 (±0.20)	68 (±3)	0.74 (±0.03)	234 (±26)	226 (±26)
Triton X-100	−	7.45 (±0.20)	101 (±5)	0.75 (±0.03)	337 (±40)	247 (±40)

[a] Values of $s_{20,w}$ and the Stokes radius (a) are the average of at least two determinations, with the number in parentheses the average deviation from the mean. Partial specific volumes ($\bar{\nu}_c$) and molecular weights ($M_{complex}$) were calculated from the average values of $s_{20,w}$ and a as described under Materials and Methods. The limits of $\bar{\nu}_c$ are shown in parentheses while the error for $M_{complex}$ was estimated by statistical procedures as described in Waser (1966). [b] The molecular weight of the protein portion of the complex was estimated by subtraction of the micellar molecular weight of the detergent (see Materials and Methods) from the molecular weight of the complex. In the case where phosphatidylcholine (PC) was also present, the amount of phosphatidylcholine bound was estimated from the partial specific volume of the complex (see Results and Discussion), and this value plus the micellar molecular weight was subtracted to give the protein molecular weight.

Assuming that each protein molecule binds one micelle of detergent (and neglecting the contribution of phosphatidylserine), a molecular weight for the protein portion of the complex of 215 000 (±21 000) can be estimated (Table I). From the difference in partial specific volume observed in the presence and absence of phosphatidylcholine, the amount of phosphatidylcholine bound can be estimated (0.23 g of phosphatidylcholine/g of protein), giving a molecular weight for the protein portion of 200 000 (±19 000), in good agreement with the value observed in the absence of phosphatidylcholine.

Table I shows the results of similar sedimentation and gel filtration analyses carried out on the (Mg^{2+})-ATPase solubilized in both octyl glucoside and Triton X-100. As expected, the size of the complex increases with the size of the detergent micelle, reaching a maximum of M_r 337 000 (±40 000) for Triton X-100 [for which the micelle size is M_r 90 000 (Hjelmeland & Chrambach, 1984)]. When the micellar molecular weight is subtracted from the complex molecular weight, a range of molecular weights for the protein portion is observed (M_r 200 000–247 000) that is within the experimental error of the determinations. The reproducibility of this value using a variety of detergents would suggest that M_r 200 000–247 000 is a reliable estimate of the molecular weight of the protein portion of the coated vesicle (Mg^{2+})-ATPase.

To test whether the detergent-solubilized (Mg^{2+})-ATPase corresponded to the ATP-hydrolyzing portion of the coated vesicle proton pump (as suggested earlier), the cholate-solubilized enzyme was reconstituted into phospholipid vesicles by dialysis (see Materials and Methods). This method has been extensively used in reconstitution of membrane proteins (Knowles & Racker, 1975; Goldin, 1977; Forgac & Chin, 1981; Goldin & Rhoden, 1978) and has the advantage of yielding very tightly sealed vesicles (Forgac & Chin, 1981). As previously reported (Xie et al., 1984), successful reconstitution of the coated vesicle proton pump required the presence of a 100000g pellet fraction added prior to reconstitution. We also observed that inclusion of cholesterol (in a weight ratio to phospholipid of 1:2) increased the proton pumping activity of the reconstituted enzyme by 8–10-fold (data not shown). Figure 2[3] shows the ATP-dependent acidification of reconstituted vesicles containing the cholate-solubilized (Mg^{2+})-ATPase obtained from sucrose density gradient sedimentation. This acidification was abolished on addition of the proton ionophore FCCP. A 3–4-fold lower activity was observed if the reconstitution was carried out with the other fractions from the sucrose gradient (this residual activity was due to the small amount of ATPase activity remaining in the 100000g pellet plus the activity not included in the two peak fractions from the sucrose gradient). As can be seen (Figure 2), the detergent-solubilized (Mg^{2+})-ATPase alone was not sufficient to reconstitute proton pumping activity. This result suggests that, in addition to the ATP-hydrolyzing complex, other components (possibly membrane-bound subunits that are not solubilized by cholate) are required to reconstitute proton transport.

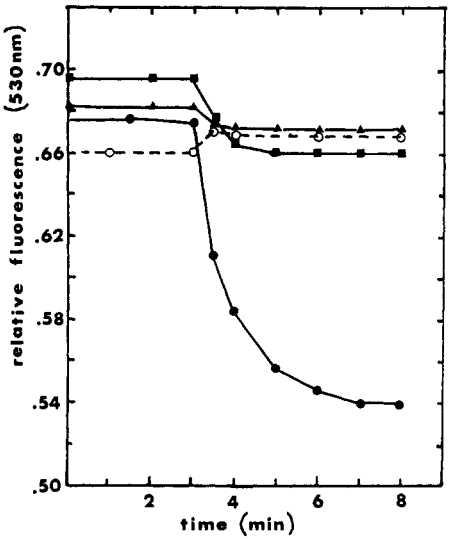

FIGURE 2: ATP-dependent acidification of reconstituted vesicles containing cholate-solubilized (Mg^{2+})-ATPase obtained from sucrose density gradient sedimentation. Solubilization and sedimentation were performed as described in Figure 1a. The peak of ATPase activity (fractions 5 and 6) was reconstituted into phospholipid vesicles by dialysis either alone (▲) or with addition of a 100000g pellet fraction (●) (see Materials and Methods). Alternatively, the remaining gradient fractions were pooled, concentrated, and reconstituted with the 100000g pellet (■). Reconstituted vesicles were then diluted into solubilization buffer containing 2 μM acridine orange and 3.3 μM valinomycin,[3] and the fluorescence intensity at 530 nm (excitation at 490 nm) was measured at 23 °C. At 3 min, 1 mM ATP and 2 mM $MgSO_4$ were added. Vesicles containing the peak of ATPase activity plus the 100000g pellet were also assayed in the presence of 5 μg/mL FCCP (○).

To further establish the relation between the detergent-solubilized (Mg^{2+})-ATPase and the reconstituted proton pump, the inhibitor sensitivity of the two activities was compared (Table II). As previously observed for the proton pump in intact coated vesicles (Forgac et al., 1983; Forgac & Cantley, 1984), (Mg^{2+})-ATPase activity and proton pumping were resistant to the (Na^+,K^+)-ATPase inhibitor ouabain but were sensitive to the alkylating agent NBD-Cl and the sulfhydryl reagent NEM. These results provide further evidence that the detergent-solubilized (Mg^{2+})-ATPase studied corresponds to the ATP-hydrolyzing portion of the coated vesicle proton pump.

[3] Valinomycin was included in the proton transport assay in order to prevent the generation of a membrane potential (inside positive) which would oppose further proton transport.

Table II: Inhibitor Sensitivity of Gradient-Purified (Mg^{2+})-ATPase Activity and Proton Transport in Reconstituted Vesicles[a]

inhibitor	concentration	relative (Mg^{2+})-ATPase activity	relative H^+ transport
none	-	1.00 (±0.02)	1.00 (±0.04)
ouabain	100 μM	0.96 (±0.02)	0.98 (±0.04)
NBD-Cl	10 μM	0.10 (±0.03)	0.07 (±0.05)
	100 μM	0.00 (±0.01)	0.01 (±0.02)
NEM	100 μM	0.15 (±0.04)	0.10 (±0.06)
	1.0 mM	0.01 (±0.01)	0.00 (±0.02)

[a] (Mg^{2+})-ATPase activity of cholate-solubilized enzyme after sucrose density gradient sedimentation was assayed in the presence of detergent (plus phospholipid) as described under Materials and Methods. ATP-dependent proton transport in reconstituted vesicles containing the cholate-solubilized, gradient purified (Mg^{2+})-ATPase (plus the 100000*g* pellet fraction) was assayed with acridine orange as described in the same section. Incubation of the solubilized enzyme or reconstituted vesicles with the indicated concentrations of NBD-Cl or NEM was carried out for 10 min at 23 °C. Where indicated, ouabain (100 μM) was added directly to the ATPase assay or was included in the reconstitution buffer (thus allowing it access to any sequestered ouabain binding sites).

It is of interest to compare the detergent-solubilized coated vesicle ATPase with other detergent-solubilized cation pumps. In both Triton X-100 (Clarke, 1975) and $C_{12}E_8$ (Brotherus et al., 1981), the (Na^+,K^+)-ATPase has a molecular weight of 140000–170000, corresponding to an α–β dimer (Forgac & Chin, 1985), the $C_{12}E_8$ complex possessing ATPase activity. Similarly, $C_{12}E_8$-solubilized (Ca^{2+})-ATPase from sarcoplasmic reticulum has a molecular weight of 127000 (Dean & Tanford, 1978), again indicating a single catalytic subunit per detergent micelle. There is, nevertheless, considerable evidence that both of these cation pumps form oligomers in the membrane of the structure $\alpha_2\beta_2$ [for the (Na^+,K^+)-ATPase] or α_2 [for the (Ca^{2+})-ATPase] (Forgac & Chin, 1985). Whether the molecular weight of the coated vesicle ATPase (M_r 200000–247000) corresponds to a dimer of tightly associated catalytic subunits of molecular weight similar to that observed for the (Na^+,K^+)- and (Ca^{2+})-ATPase (M_r 100000–120000) or to some other structure must await determination of the subunit composition. The ATP-hydrolyzing portion (F_1) of the mitochondrial (H^+)-ATPase has a molecular weight of approximately 360000 (Senior, 1973) but differs from the ATP-hydrolyzing portion of the coated vesicle pump in that it is not an integral membrane protein. The nature and role of other proteins or lipids required for reconstitution of proton transport by the coated vesicle pump are currently under investigation.

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Registry No. ATPase, 9000-83-3; Triton X-100, 9002-93-1; H^+, 12408-02-5; sodium cholate, 361-09-1; octyl glucoside, 29836-26-8.

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