J. Biochem. 120, 608-615 (1996)

Properties of H^+-ATPase from Rat Liver Lysosomes as Revealed by Reconstitution into Proteoliposomes[1]

Masayuki Okamoto, Norikazu Hiratani, Kunizo Arai, and Shoji Ohkuma[2]

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Ishikawa 920

Received for publication, April 30, 1996

The properties of H^+-ATPase from rat liver lysosomes were analyzed by reconstituting proton pump activity from solubilized enzyme and *Escherichia coli* phospholipids in proteoliposomes devoid of anion-channels. The reconstitution procedure involved solubilization of the ATPase with *n*-octyl-β-D-thioglucoside in the presence of asolectin, and incorporation of the solubilized enzyme into *E. coli* phospholipid liposomes by dilution, freeze-thawing, and sonication. Proton pump activity of reconstituted H^+-ATPase as detected by the ATP-dependent quenching of acridine orange fluorescence indicated that ATP can be replaced with dATP and to a lesser extent with GTP, but not with any other nucleotide, that Mg^{2+} can be replaced with Mn^{2+}, but not with Ca^{2+}, Sr^{2+}, or Ba^{2+}, that Zn^{2+}, Pd^{2+}, Cd^{2+}, and Hg^{2+} were inhibitory, and that the enzyme was sensitive to inhibitors of v-type H^+-ATPase, including bafilomycin A_1, *N*-ethylmaleimide, DCCD, DIDS, and tri-*n*-butyltin. The enzyme showed unique sensitivity to anions and was activated by chloride, fluoride, and bromide from inside, but not from outside the vesicles. It was inhibited by sulfate, sulfite, and thiocyanate from outside the vesicles, and by nitrate from both inside and outside the vesicles.

Key words: anion-sensitivity, H^+-pump, lysosome, reconstitution, vacuolar H^+-ATPase.

An Mg^{2+}-ATP-dependent electrogenic proton pump (H^+-ATPase) is involved in the maintenance of acidic pH within lysosomes (*1, 2*). We purified H^+-ATPase from rat liver lysosomes (*3*). The lysosomal H^+-ATPase shared similar properties to those of other v-type H^+-ATPases, except for its anion sensitivity, which was similar to that of the enzymes from chromaffin granules (*4, 5*), plant tonoplasts (*6*), and yeast (*7*), but not to that of the enzymes from kidney (*8*) and coated vesicles (*9*).

The anion-sensitivity of the lysosomal proton pump needs further study. Chloride, for example, is generally considered to provide the conductance necessary to support continuous acidification by dissipating the membrane potential established by an electrogenic proton pump. In fact, the coexistence of v-type H^+-ATPase and anion channel (*e.g.*, chloride) or transporter (*e.g.*, sulfate) is a common feature of endomembrane compartments (*10*). However, the ATPase activity of purified lysosomal H^+-ATPase was itself activated by chloride. Furthermore, there are some discrepancies between the effects of anions on ATP hydrolysis and on proton pump activity. Fluoride activates H^+-ATPase but not H^+-transport, whereas nitrate, sulfate, and sulfite inhibit purified H^+-ATPase but support proton pump activity of intact lysosomes (*3*). Therefore, whether the proton-translocating activity of lysosomal H^+-ATPase itself is also sensitive to anions should be studied. However, it is difficult to analyze the anion sensitivity of the proton pump itself on intact lysosomes, because of the presence of anion channels (or carriers) on the same membranes that affect intravesicular acidification. More precise studies using a reconstitution system devoid of these anion transporters are required to clarify the regulatory actions of these anions on lysosomal H^+-ATPase.

Here, we report the properties of lysosomal H^+-ATPase with special emphasis on its anion-sensitivity as revealed from a reconstitution study using proteoliposomes devoid of anion transporter(s).

MATERIALS AND METHODS

Materials—Triton WR-1339 was obtained from Ruger Chemical (Irvington, NJ). *n*-Octyl-β-D-thioglucoside was purchased from Dojin (Kumamoto). Acridine orange was supplied by Wako (Tokyo). *Escherichia coli* phospholipid was obtained from Avanti Polar Lipids (Alabaster, AL). *E. coli* phospholipid was purified as described by Newman and Wilson (*11*). Bafilomycin A_1 was supplied by Dr. K.

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-762-8151 (Ext. 6411), FAX: +81-762-34-4462, e-mail, ohkuma@kenroku.ipc.kanazawa-u.ac.jp

Abbreviations: AMP-CPP, α,β-methylene adenosine 5′-triphosphate; AMP-PCP, β,γ-methylene adenosine 5′-triphosphate; Bicine, *N,N*-bis(2-hydroxylethyl)glycine; BSA, bovine serum albumin; DCCD, *N,N′*-dicyclohexylcarbodiimide; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DTT, dithiothreitol; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazone; FD, fluorescein dextran; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino) propanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole; NEM, *N*-ethylmaleimide; TMAH, tetramethylammonium hydroxide; TMG, tritosomal membrane ghosts.

Altendorf (University of Osnabrück, Osnabrück, Germany). The other chemicals used were of analytical grade and were mostly obtained from Sigma (St. Louis, MO).

Reconstitution of H⁺-ATPase—Triton-filled lysosomes (tritosomes) and their membranes (tritosomal membrane ghosts, TMG) were prepared as described, with slight modifications (*3*). The livers were perfused well with 0.25 M sucrose, 1 mM EDTA, and 0.1% ethanol before excision, which resulted in a tritosome preparation with a relative (to homogenate) β-*N*-acetyl-D-glucosaminidase specific activity close to 80. Protease inhibitors (pepstatin A, chymostatin, leupeptin, and antipain, all at 5 μg/ml) were included in every solution used to prepare tritosomes and membrane ghosts. To prepare the latter, tritosomes were added by pipette to 10 volumes of chilled 20 mM sucrose buffer containing 10 mM Mops/tetramethylammonium hydroxide (TMAH) (pH 7.0), 1 mM $MgSO_4$, 2 mM dithiothreitol (DTT), 5 mg/ml bovine serum albumin (BSA), and the protease inhibitors. After stirring for 10 min, the suspension was centrifuged at 30,000 × *g* for 15 min and the resultant pellet was washed three times with 0.25 M sucrose containing 20 mM Mops/TMAH (pH 7.0), 0.5 mM EDTA, 1 mM DTT, and the protease inhibitors.

TMG were solubilized with octylthioglucoside as described before (*3*). The TMG suspension which had been adjusted to 1 mg protein/ml in the solubilization buffer [20 mM Mops/TMAH (pH 7.0), 10 mM DTT, 20% (v/v) glycerol, 1 mM EDTA, and 5 μg/ml each of protease inhibitors] was mixed with 0.2 mg/ml asolectin and with 1.0% (w/v) *n*-octyl-β-D-thioglucoside. Immediately thereafter, the mixture was vortexed for 15 s, incubated on ice for 10 min, and then sedimented at 105,000 × *g* for 1 h. The resulting supernatant was used as the solubilized fraction. The solubilized H⁺-ATPase was then mixed with sonicated phospholipids (protein/lipid ratio = 1/20) and *n*-octyl-β-D-thioglucoside [final concentration of 1.4% (w/v)] (*12*). After having been kept for 30 min on ice, the mixture was frozen at −80°C and thawed on ice, then injected into 30 volumes of dilution buffer [0.15 M KCl, 10 mM DTT, 1 mM EDTA, 20 mM Mops/TMAH (pH 7.0), and 5 μg/ml each of protease inhibitors], warmed to 30°C. The whole was centrifuged at 105,000 × *g* for 1 h. The resulting pellet (proteoliposomes) was resuspended in washing buffer [0.15 M KCl, 10 mM DTT, 20 mM Mops/TMAH (pH 7.0), protease inhibitors], and used in the proton transport assay directly or after sonication in a bath type sonifier for 15 s. For some studies of the sidedness of the effect of anions, KCl in the dilution and washing buffers was replaced with an appropriate salt (for example, K-gluconate).

Measurement of Proton Transport and ATP Hydrolysis—The formation of a pH gradient (inside acid) was measured by utilizing of the fluorescence quenching of the permeant basic dye, acridine orange (reconstituted proteoliposomes), and fluorescein dextran (FD) incorporated into lysosomes (intact lysosomes). The formation of a membrane potential gradient (inside positive) was measured in terms of fluorescence quenching of diS-C_3(5) (intact lysosomes). The assay buffer contained 0.15 M KCl (or an appropriate salt), 20 mM Hepes/TMAH (pH 7.5 or 7.0), 0.5 mM (or 2.5 mM) ATP-Mg (or ATP-NA_2 + $MgCl_2$) in a final volume of 2.0 ml with or without the dyes. The concentrations of the dyes were 1 μM for acridine orange and 0.5 μM for diS-C_3(5). Fluorescence was measured at 30°C or 37°C with a Hitachi 310 spectrofluorometer, with excitation at 480 nm and emission at 540 nm for acridine orange, and at 620 and 670 nm for diS-C_3(5), respectively. Though the reconstituted lysosomal H⁺-ATPase showed only several percent of the total activity, the effects of anions on the proton pump activity were observed reproducibly and essentially the same results were obtained in two or three separate experiments. ATPase activity was measured as described (*3*).

Other Analytical Methods—Protein was determined by the amido black/solid phase method of Schaffner and Weissmann using BSA as the standard (*13*).

RESULTS

1. Reconstitution of Lysosomal H⁺-ATPase—Lysosomal H⁺-ATPase was reconstituted by adapting the procedure of D'Souza *et al.* (*14*). We used *n*-octyl-β-D-thioglucoside instead of *n*-octylglucoside to solubilize the ATPase. There are two critical points for the successful reconstitution of the lysosomal H⁺-ATPase. One is the concentration of the detergent. As shown in Fig. 1, the activity of the reconstituted enzyme was maximal when the detergent concentration of the mixture was 1.4% (w/v). The other is the source of the lipid used for reconstitution. As shown by D'Souza *et al.*, *E. coli* phospholipid yielded 3-fold better results than asolectin, which has generally been used in reconstitution studies. The obtained proteoliposome showed a twofold enrichment of bafilomycin A_1-sensitive ATPase activity which was enriched by a factor of two (142 mU/mg protein) in the proteoliposomes, as compared with the original membrane extract (77 mU/mg protein), indicating selective reconstitution of H⁺-ATPase.

The reconstituted proteoliposomes showed rapid, ATP-dependent acidification, as revealed by the quenching of acridine orange fluorescence, especially when proteoliposomes were prepared without sonication (Fig. 2, a and b). In K-gluconate medium, this quenching was absolutely depen-

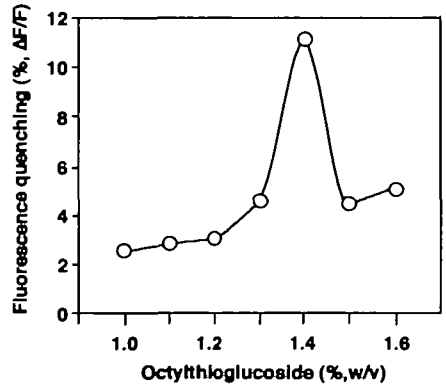

Fig. 1. **Effect of *n*-octyl-β-D-thioglucoside on reconstitution of the proton pump.** An *n*-octyl β-D-thioglucoside extract of TMG was mixed with liposomes of *E. coli* phospholipid (protein/phospholipid ratio = 1/20) at the designated concentration of *n*-octyl-β-D-thioglucoside. After incubation on ice for 30 min and one cycle of freeze-thawing, the mixture was injected into 30 volumes of dilution buffer [20 mM Mops/TMAH (pH 7.0), 0.15 M KCl, 10 mM DTT, 1 mM EDTA, and 5 μg/ml each of protease inhibitors]. The other procedures and measurement of acidification were as described in "MATERIALS AND METHODS."

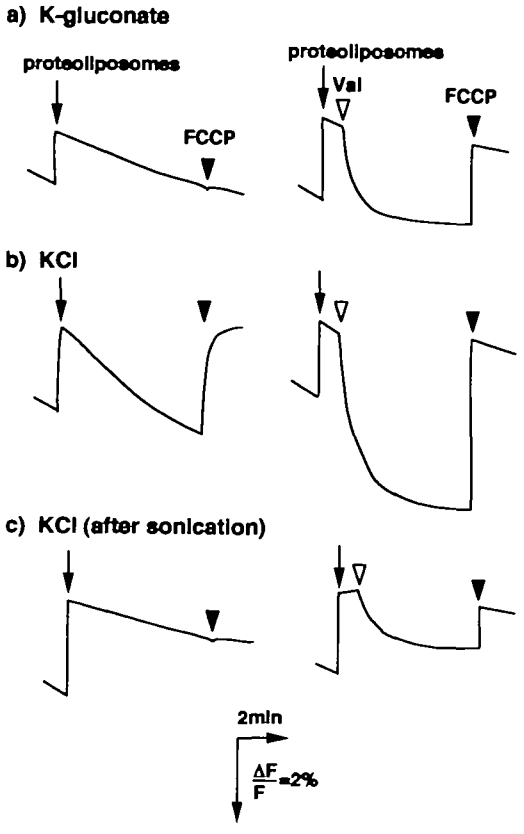

Fig. 2. **Fluorescence quenching of acridine orange by the internal acidification of reconstituted proteoliposomes.** Proteoliposomes were prepared in K-gluconate buffer [20 mM Mops/TMAH (pH 7.0), 0.15 M K-gluconate, 10 mM DTT, and 5 μg/ml each of protease inhibitors]. Reconstituted proteoliposomes were incubated at 37°C in assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 2.5 mM ATP-Mg, 1 μM acridine orange, and 0.15 M of the indicated potassium salt. (a) 0.15 M K-gluconate, (b) 0.15 M KCl, (c) 0.15 M KCl, proteoliposomes were dispersed using a bath type sonicator for 15 s. Additions: (↓) proteoliposome (50 μl), (▽) Val (valinomycin) (0.1 μM), (▼) FCCP (carbonylcyanide-4-trifluoromethoxyphenylhydrazone) (0.5 μM).

TABLE I. **Substrate specificity of the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 0.15 M KCl, 0.5 mM $MgCl_2$, 1 μM acridine orange, and 0.5 mM of the indicated substrate.

Substrate	Proton pump activity (% of control)
ATP (control)	100
dATP	88
GTP	13
ITP	0
UTP	0
CTP	0
ADP	1
AMP-CPP	1
AMP-PCP	2
AMP	0
cAMP	2
Pyrophosphate	0
ADP+ATP	27
AMP-CPP+ATP	58
AMP-PCP+ATP	57
cAMP+ATP	71
Pyrophosphate+ATP	60

TABLE II. **Effect of divalent cations on the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 0.15 M KCl, 0.5 mM ATP, 1 μM acridine orange, and 0.5 mM of the indicated divalent cations.

Divalent cation (Cl salt)	Proton pump activity (% of control)
Mg^{2+} (control)	100
Mn^{2+}	77
Ca^{2+}	9
Sr^{2+}	9
Ba^{2+}	9
$Ca^{2+}+Mg^{2+}$	99
$Sr^{2+}+Mg^{2+}$	104
$Ba^{2+}+Mg^{2+}$	99
$Zn^{2+}+Mg^{2+}$	0
$Pd^{2+}+Mg^{2+}$	0
$Cd^{2+}+Mg^{2+}$	2
$Hg^{2+}+Mg^{2+}$	9
No addtion	9
EDTA-Na_2	0

TABLE III. **Effect of various inhibitors upon the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 0.15 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, and 1 μM acridine orange.

Inhibitor	Concentration	Proton pump activity (% of control)
No addition (control)	–	100
Bafilomycin A_1	100 nM	0
NEM	1 mM	0
NBD-Cl	25 μM	36
DCCD	25 μM	34
DIDS	10 μM	5
SITS	25 μM	39
Tri-*n*-butyltin	5 μM	9
Ouabain	100 μM	100
Vanadate	100 μM	78
Efrapeptin	5 μg/ml	97
Oligomycin	2.5 μg/ml	83
NaN_3	10 mM	48

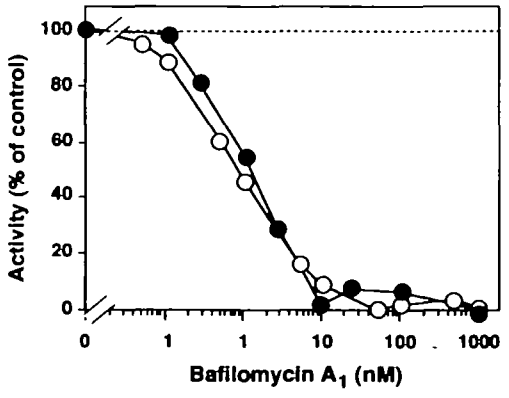

Fig. 3. **Effect of bafilomycin A_1 on the ATPase activity of purified H^+-ATPase and on the proton pump activity of reconstituted proteoliposomes.** Bafilomycin A_1-sensitive ATPase activity of Mono Q fraction (see Ref. 3) was measured after incubation at 30°C for 40 min in an assay buffer containing 40 mM Hepes/TMAH (pH 7.5), 0.1 M KCl, 0.5 mM $MgCl_2$, 0.5 mM ATP-Na_2, and the indicated concentrations of bafilomycin A_1. The proton pump activity of reconstituted proteoliposomes was measured as described in the legend to Fig. 2 and "MATERIALS AND METHODS." (○) bafilomycin A_1-sensitive ATPase; (●) reconstituted proton pump.

dent on the presence of valinomycin, and was reversed by FCCP (Fig. 2a). However in K-chloride medium, it was independent of valinomycin, although the initial rate of fluorescence quenching was weaker than in the presence of valinomycin (Fig. 2b). This suggests that chloride channels were co-reconstituted into the same proteoliposomes. The chloride channels were excluded from sonicated proteoliposomes as shown in Fig. 2c, where fluorescence quenching was absolutely dependent on valinomycin even in KCl medium. The properties of the lysosomal H⁺-ATPase were analyzed using the sonicated proteoliposomes in the following study.

2. Properties of the Reconstituted Lysosomal H⁺-ATPase—Substrate specificity: The relative effectiveness of substrates was in the order of ATP > dATP ≫ GTP. The activity of GTP was sometimes hardly detectable. ITP, UTP, CTP, ADP, pyrophosphate (PP_i), AMP, cyclic AMP, and non-hydrolyzable analogs of ATP [α,β-methylene ATP (AMP-CPP), β,γ-methylene ATP(AMP-PCP)] were all ineffective. ADP inhibited the ATP-dependent acidification (Table I). The substrate specificity of the reconstituted H⁺-pump was similar to that of the purified H⁺-ATPase. ITP and UTP (sometimes GTP) did not support H⁺-pump activity though they expressed some ATPase activity.

Divalent cation specificity: Table II shows the effect of different divalent cations on the H⁺-pump activity of the reconstituted H⁺-ATPase. The divalent cation requirement was in the order of $Mg^{2+} > Mn^{2+}$. Although Ca^{2+} expressed

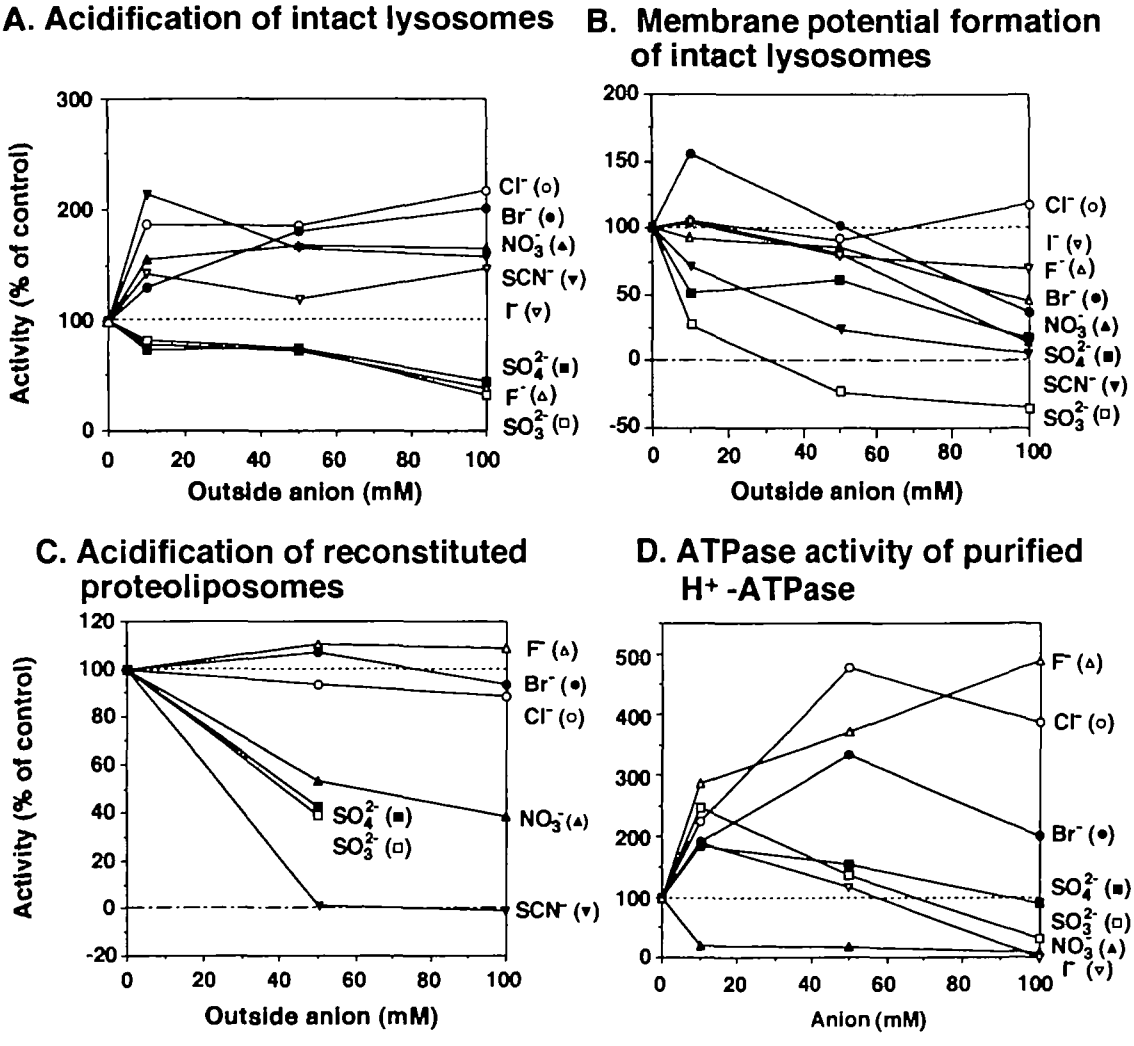

Fig. 4. **Effect of anions on the proton pump activities of intact lysosomes (A, B) and reconstituted proteoliposomes (C) and on the ATPase activities of purified H⁺-ATPase (D).** (A) Acidification of intact lysosomes. FD-loaded lysosomes were suspended in 2 ml of 20 mM Hepes/TMAH (pH 7.0), containing 2.5 mM $MgCl_2$ and 0.15 M potassium salt. Acidification was initiated by adding 2.5 mM ATP-Na_2. Initial rates of acidification were estimated from tangents of the initial parts of the curves. (B) Membrane potential formation of intact lysosomes: Dextran-loaded lysosomes were suspended in the same buffer containing 0.5 μM diS-C_3(5) and ATP was added to initiate proton transport. Membrane potential formation was estimated from the percent quenching of diS-C_3(5) fluorescence. The potassium concentration of all assay mixtures was adjusted to 0.15 M using K-gluconate. (C) Acidification of proteoliposomes: Reconstituted proteoliposomes were incubated at 37°C in the assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 2.5 mM ATP-Mg, 1 μM acridine orange, and the indicated concentration of each anion: (○) KCl; (●) KBr; (▽) KI; (△) KF; (■) K_2SO_4; (□) K_2SO_3; (▲) KNO_3; (▼) KSCN. All mixtures were adjusted to 0.15 M potassium by K-gluconate. (D) ATPase activity of purified lysosomal H⁺-ATPase: adapted from Ref. 3.

some ATPase activity, Ca^{2+}, Sr^{2+}, and Ba^{2+} were almost ineffective. Zn^{2+}, Pd^{2+}, Cd^{2+}, and Hg^{2+} were inhibitory. Fe^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} affected acridine orange fluorescence and therefore their results were difficult to evaluate.

Effect of inhibitors: The effects of various inhibitors on the reconstituted lysosomal H^+-ATPase are shown in Table III. Bafilomycin A_1, a potent selective inhibitor of v-type ATPase (*15*), inhibited both proton translocation and ATP hydrolysis activities of reconstituted proteoliposomes, with an IC_{50} of about 1 nM (Fig. 3). Among other inhibitors that inhibit purified lysosomal H^+-ATPase, NEM and tri-*n*-butyltin completely inhibited the H^+-pump activity of proteoliposomes. DCCD, a powerful inhibitor of purified H^+-ATPase, only moderately affected the reconstituted H^+-pump activity. This may be partly due to the shorter incubation period or the larger amount of lipids in the proteoliposome preparation, which would reduce its effect. NBD-Cl and SITS only partially inhibited the acidification of reconstituted proteoliposomes, although they inhibited purified H^+-ATPase. Ouabain, vanadate, efrapeptin, and oligomycin were all without effect. Sodium azide, which had little effect on the purified H^+-ATPase, inhibited H^+-pump activity. This may be due to the uncoupling effect of the lipophilic azide anion at high concentrations. So far, the reconstituted proton pump showed properties that are shared not only by the purified enzymes, but also by the proton pump on intact lysosomes.

3. Effect of Anions on the Lysosomal H^+-ATPase—We compared the effects of anions on the proton pump activities of reconstituted proteoliposomes with those of the intact lysosomes and with the ATPase activity of the purified H^+-ATPase. The effect of extravesicular anions on ATP-driven H^+-uptake into the intact lysosomes is shown in Fig. 4A. Chloride, bromide, nitrate, and thiocyanate supported the H^+-uptake, but fluoride, sulfate, and sulfite did not. The membrane potential formation of intact lysosomes was inhibited by sulfate, sulfite, thiocyanate, and nitrate (at high concentration) but was hardly affected by other anions (chloride, bromide, iodide, and fluoride) (Fig. 4B). The proton pump activity of the reconstituted proteoliposomes devoid of anion channel was not stimulated by chloride, bromide, nitrate, or thiocyanate (Fig. 4C, inside K-gluconate). These results show that stimulation of the H^+-pump increases with increasing permeability of the anions and, hence, with their ability to achieve charge compensation for pumped hydrogen ions. But, as mentioned in the introduction, chloride, bromide, and fluoride activated H^+-ATPase (Fig. 4D). The results suggest that chloride and bromide activate lysosomal acidification from inside the liposomal vesicles, but not from outside, and also that fluoride has some additional inhibitory effect on the lysosomal proton pump (acidification) that requires the presence of some factors (*e.g.*, channels or transporters) other than the H^+-ATPase. The other anions (sulfite, sulfate, thiocyanate, and nitrate) were inhibitory to reconstituted proteoliposomes, suggesting that these anions have inhibitory binding sites on the exterior of the H^+-ATPase and that thiocyanate and nitrate accelerate lysosomal acidification by dissipating membrane potential due to their permeation through lysosomal membranes. To analyze these effects of anions, we studied proteoliposomes loaded inside with various concentrations of different anions.

4. Sidedness of the Effect of Anions on Reconstituted Proton Pump—The advantage of a reconstitution study is that the intravesicular contents, such as anion species and concentration, can be manipulated by incorporating the anions in question while preparing the proteoliposomes. To examine the effects of anions in more detail, we analyzed the sidedness of their effects using this property. The proton pump activity of reconstituted proteoliposomes was higher when chloride, bromide or fluoride was incorporated inside. On the other hand, extravesicular anions did not enhance, but rather inhibited with increasing concentration, the proton pump activity of proteoliposomes loaded with the same anions inside (Fig. 5). These results confirmed that chloride, bromide, and fluoride activate H^+-ATPase from inside proteoliposomes. When proteoliposomes were prepared in K-gluconate buffer, extravesicular

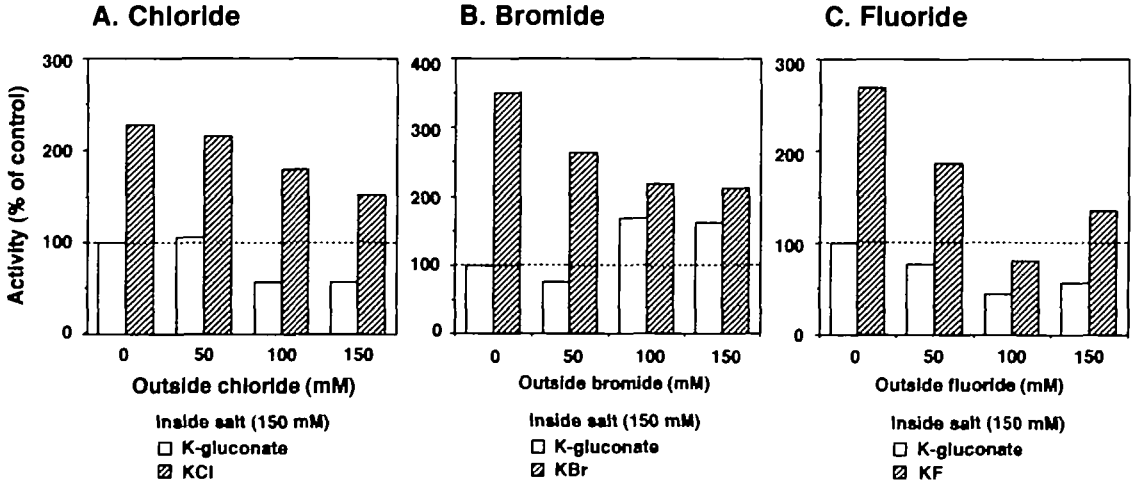

Fig. 5. **Effect of (A) chloride, (B) bromide, and (C) fluoride on the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in the assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 2.5 mM ATP-Mg, 1 μM acridine orange, and the indicated concentration of each anion: (A) KCl, (B) KBr, and (C) KF. All assay mixtures were adjusted to 0.15 M potassium by K-gluconate. Insert: open bar, proteoliposomes containing K-gluconate; hatched bars, proteoliposomes containing (A) KCl, (B) KBr, or (C) KF. Potassium salt (0.15 M) was included in the dilution buffer to load the proteoliposomes with the anion under study.

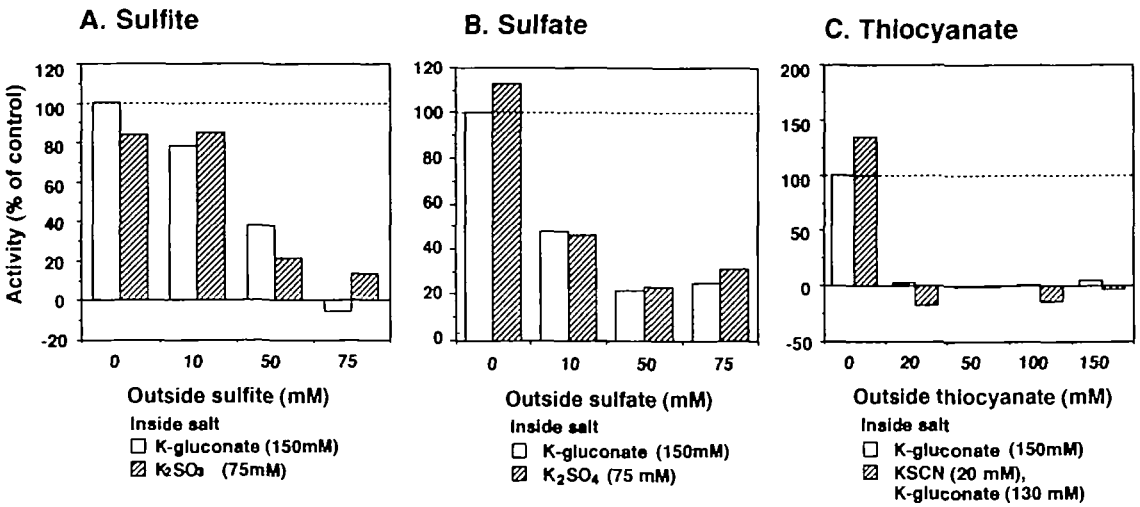

Fig. 6. **Effects of (A) sulfite, (B) sulfate, and (C) thiocyanate on the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in the assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 2.5 mM ATP-Mg, 1 μM acridine orange, and the indicated concentration of each anion: (A) K_2SO_3, (B) K_2SO_4, or (C) KSCN. All assay mixtures were adjusted to 0.15 M potassium with K-gluconate. Insert: open bar, proteoliposomes containing K-gluconate; hatched bars, proteoliposomes containing (A) K_2SO_3, (B) K_2SO_4, or (C) KSCN.

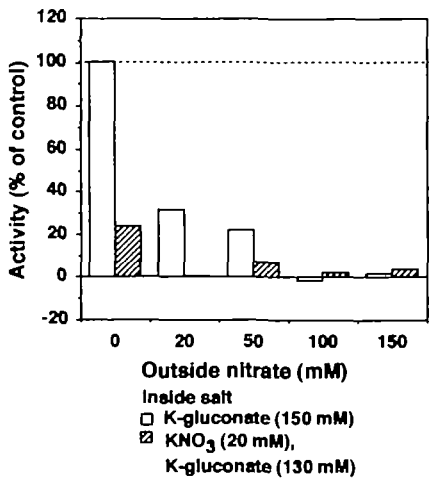

Fig. 7. **Effect of nitrate on the proton pump activity of reconstituted proteoliposomes.** Reconstituted proteoliposomes were incubated at 37°C in the assay buffer containing 20 mM Hepes/TMAH (pH 7.5), 2.5 mM ATP-Mg, 1 μM acridine orange, and the indicated concentration of KNO_3. All assay mixtures were adjusted to 0.15 M potassium with K-gluconate. Insert: open bar, proteoliposomes containing K-gluconate; hatched bar, proteoliposomes containing KNO_3.

chloride, fluoride, and bromide inhibited the proton pump activity (Fig. 5).

Figure 6 shows the results of similar experiments with sulfate, sulfite, and thiocyanate, which inhibit purified H⁺-ATPase activity. Proteoliposomes with intravesicular sulfate, sulfite, and thiocyanate expressed similar proton pump activity to those without these anions (namely, K-gluconate inside). However, these anions, when present outside the vesicles, suppressed proton pump activity with increasing concentration, irrespective of the presence or absence of the same anions within the vesicles, suggesting that these anions exert their proton pump inhibitory effect from outside the vesicles. Thiocyanate had the strongest activity among these anions and severely inhibited the proton pump activity at concentrations 20 mM above that on the outside.

In contrast, nitrate (20 mM) incorporated into vesicles inhibited most (80%) of the proton pump activity. Extravesicular nitrate (20 mM) also inhibited (>70%) the proton pump activity (Fig. 7). Both nitrate and thiocyanate, however, supported the proton pump (acidification) of intact lysosomes as described above (Fig. 4A). This is probably due to their high permeation through anion channel(s) present on lysosomal membranes.

DISCUSSION

Effects of various anions on vacuolar ATPase have been reported by several investigators in vacuolar organelles other than lysosomes. Although chloride functions primarily by dissipating the electrochemical gradient of protons across the vacuolar membrane created by electrogenic proton transport, direct stimulation of H⁺-ATPase by chloride and bromide has also been reported in several vacuoles (*16, 17*). Moriyama and Nelson reported that chromaffin granule ATPase has internal anion binding sites for these ions and that it may regulate the proton pumping activity of the H⁺-ATPase (*5*). On the other hand, nitrate, sulfate, and sulfite, which are inhibitory to the chromaffin granules and yeast vacuole H⁺-ATPases (*18-20*), inhibit binding of ATP on the A subunit of the H⁺-ATPase (*21*), suggesting that these oxyanions regulate the vacuolar H⁺-ATPase through binding to these sites.

We showed that the lysosomal proton pump requires membrane-permeant anions for maximum acidification: this is partly because continued internal acidification requires dissipation of the membrane potential difference that is generated by the electrogenic H⁺-ATPase. This is attained by the anion (chloride) channel present on the lysosomal membrane (*22*). On the other hand, we also showed that purified lysosomal H⁺-ATPase itself is sensitive to anions, being maximally activated by chloride and

inhibited by nitrate (*3*). The latter findings suggested that the anion-sensitivity of the lysosomal proton pump is due to the anion-sensitivity of H^+-ATPase itself. Therefore, we investigated the effect of anions on the H^+-translocating activity of H^+-ATPase in reconstituted proteoliposomes devoid of other ion channels, prepared by reconstitution using dilution-freeze/thawing and sonication.

The reconstituted proton pump showed properties that were similar to those of the purified enzyme, including substrate specificity, divalent cation requirement, and inhibitor sensitivity. The sensitivity of reconstituted proton pump to azide might be explained by the uncoupling effect of azide. Some other slight differences may have originated from the lower sensitivity of the acidification assay of proteoliposomes; for example, the very low ability of GTP, ITP, and UTP to support H^+-pump activity, despite some hydrolysis by the purified enzyme.

On the other hand, the anion-sensitivity of the reconstituted proton pump was similar to that of the purified ATPase only for some types of ions (*e.g.*, nitrate and thiocyanate). Contrary to their effect on the purified ATPase, chloride, bromide, and fluoride did not activate the proton pump activity of proteoliposomes. Studies on the sidedness of the effect of anions showed that they activated the proton pump activity of proteoliposomes only from inside the vesicles. These results showed that the various anions can be grouped into three: (i) those activating only from inside the vesicles (chloride, fluoride, and bromide), (ii) those inhibiting from outside the vesicles (sulfite, sulfate, and thiocyanate), and (iii) those inhibiting from both sides of the vesicles (nitrate).

The mode of activation of the proton pump of reconstituted proteoliposomes by anions suggests that H^+-ATPase has several anion-binding sites accessible either from outside, from inside or from either side of the membranes (Fig. 8). (i) Binding sites for sulfate and sulfite (and chloride, fluoride, though the effects are weak) are on the externally exposed portion of the H^+-ATPase, (ii) those for chloride, bromide, and fluoride are on the internally exposed site of H^+-ATPase, and (iii) those for nitrate are on the membrane-embedded portion of the H^+-ATPase, as its proton pump inhibitory effect occurs from either side. Alternatively, nitrate may have interaction sites on both externally and internally exposed regions of H^+-ATPase in the vesicles. The existence of anion (nitrate) binding sites within vesicles was also suggested in chromaffin granule H^+-ATPase (*5*).

There are some discrepancies in the anion-sensitivity of proton pump activity between reconstituted proteoliposomes and intact lysosomes. Even anions that are inert (or slightly inhibitory, such as chloride and bromide) or inhibitory (such as nitrate, iodide, and thiocyanate) to the proton pump activity of reconstituted proteoliposomes can support that of intact lysosomes. Fluoride is also inert (from outside) to the proton pump activity of reconstituted proteoliposomes but inhibitory to that of intact lysosomes. Although the precise mechanism remains to be investigated, some of these discrepancies may be explained either by the absence in the reconstituted proteoliposomes of an ion channel(s) that is normally associated with lysosomal membrane, or by a difference in the environment surrounding the H^+-ATPase (including the state of H^+-ATPase in the membranes and membrane lipid compositions). For example, the different effect of fluoride on the proton pump activities between intact lysosomes and reconstituted proteoliposomes might be related to some unknown inhibitory effects of fluoride on lysosomal acidification that depends on some factors [*e.g.*, anion channel(s) or H^+-coupled transporters] other than the H^+-ATPase on lysosomal membranes. Likewise, anions (*e.g.*, nitrate, iodide, or thiocyanate) that inhibit purified ATPase and the reconstituted proton pump (from inside the vesicle) supported the proton pump activity of intact lysosomes, most probably because membranes of intact lysosomes possess anion channel(s) or transporter(s) that allow continuous acidification driven by H^+-ATPase. Otherwise, H^+-ATPase in reconstituted proteoliposomes made from *E. coli* phospholipids may be more exposed and accessible to such inhibitory anions than in intact lysosomes and accordingly may be more sensitive to them. These ions might be more permeant to liposome membranes made of *E. coli* phospholipids than to the membrane of intact lysosomes and accordingly more harmful to H^+-ATPase. Purified lysosomal ATPase is sensitive to the lipid environment and it expresses less activity with phosphatidylinositol and phosphatidic acid, but reasonable activity with other acidic phospholipids (*3*).

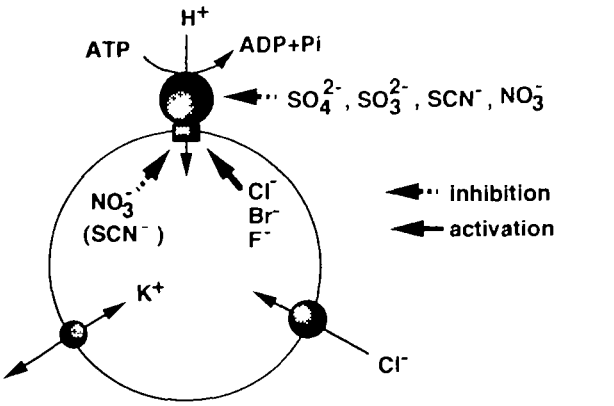

Fig. 8. **Schematic model of anion-binding sites on the proton pump.**

Recently, Dschida and Bowman have reported that nitrate acts as an oxidizing agent towards the enzyme and promotes the formation of disulfide bonds (*23*), with its inhibitory effect on the ATPase activity being specific for the vacuolar ATPase. Since thiocyanate is also a moderately strong oxidant, part of the inhibitory effect of nitrate and thiocyanate on the lysosomal H^+-ATPase might be due to the formation of a disulfide bond.

The factors that determine the lysosomal pH include the amount of H^+-ATPase on the lysosomal membranes. Especially important is the molecular form of the H^+-ATPase (whether it is present on lysosomal membranes with its full or a partial subunit composition). The V_o portion of H^+-ATPase is also located on lysosomal membranes, which may be related to the acidification activity of the lysosomal system (unpublished observation). A regulatory mechanism of the intracellular pH controlled by the detachment and attachment of the V_1 portion of H^+-ATPase on the plasma membranes has recently been reported (*24*).

One of the other factors is the membrane potential

difference across membranes. This may be regulated by conductance for chloride (*25, 26*) or other anions (*27, 28*), by the electrogenic Na^+/K^+-ATPase associated with endosome membranes (*29-31*), by the cation permeabilities (*32*) or by proton leakage (*33*). It has also been reported that the regulation of chloride conductance by protein kinase A may play a role in the control of endosomal acidification (*25*). The same mechanism might exist in lysosomes, though the lysosomal chloride channel reportedly differs from CFTR (*34*). We obtained findings that suggest the presence of potassium channel activity on lysosomal membranes from rat liver (unpublished results). Most endocytic vesicles appear to have chloride, potassium, and proton conductance and variable levels of H^+-ATPase, all of which may affect the intra-vesicular acidification generated by electrogenic H^+-ATPase (*22, 32, 34*). Further studies are required however, to clarify the roles of chloride and potassium channels in lysosomal acidification using the reconstitution system.

The role of chloride in the activation of H^+-ATPase within intact cells is notable. When the chloride concentration in proteoliposomes was 20 mM, there was little activation of the proton pump activity. But if it was raised to 50 mM, the proton pump activity was maximally expressed. Whether or not lysosomes possess this concentration of chloride ion internally, should be examined.

We are now investigating the disposition and control of H^+-ATPase subunits on the intracellular membranes, as well as the role of ions and their channels in the maintenance of intralysosomal pH.

We are grateful to Professor K. Altendorf (Osnabrück University, Germany) for the generous gift of bafilomycin A_1.

REFERENCES

1. Ohkuma, S., Moriyama, Y., and Takano, T. (1982) Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. *Proc. Natl. Acad. Sci. USA* **89**, 2758-2762
2. Moriyama, Y., Ohkuma, S., and Takano, T. (1984) Proton translocating ATPase in lysosomal membrane ghosts. Evidence that alkaline Mg^{2+}-ATPase acts as a proton pump. *J. Biochem.* **95**, 955-1007
3. Arai, K., Shimaya, A., Hiratani, N., and Ohkuma, S. (1993) Purification and characterization of lysosomal H^+-ATPase: An anion-sensitive v-type H^+-ATPase from rat liver lysosomes. *J. Biol. Chem.* **268**, 5649-5660
4. Moriyama, Y. and Nelson, N. (1987) The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. *J. Biol. Chem.* **262**, 9175-9180
5. Moriyama, Y. and Nelson, N. (1987) Internal anion binding site and membrane potential dominate the regulation of proton pumping by the chromaffin granule ATPase. *Biochem. Biophys. Res. Commun.* **149**, 140-144
6. Bennett, A.B. and Spanswick, R.M. (1983) Solubilization and reconstitution of an anion-sensitive H^+-ATPase from corn roots. *J. Membr. Biol.* **75**, 21-31
7. Kibak, H., Eeckhout, D.V., Cutler, T., Taiz, S.L., and Taiz, L. (1993) Sulfite both stimulates and inhibits the yeast vacuolar H^+-ATPase. *J. Biol. Chem.* **268**, 23325-23333
8. Wang, Z.-Q. and Gluck, S. (1990) Isolation and properties of bovine kidney brush border vacuolar H^+-ATPase: A proton pump with enzymatic and structural differences from kidney microsomal H^+-ATPase. *J. Biol. Chem.* **265**, 21957-21965
9. Arai, H., Pink, S., and Forgac, M. (1989) Interaction of anions and ATP with the coated vesicle proton pump. *Biochemistry* **28**, 3075-3082
10. Mellman, I., Fuchs, R., and Helenius, A. (1986) Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55**, 663-700
11. Newman, M.J. and Wilson, T.H. (1980) Solubilization and reconstitution of the lactose transport system from *Escherichia coli*. *J. Biol. Chem.* **255**, 10583-10586
12. Saito, S. and Tsuchiya, T. (1984) Characteristics of *n*-octyl β-D-thioglucopyranoside, a new non-ionic detergent useful for membrane biochemistry. *Biochem. J.* **222**, 829-832
13. Schaffner, W. and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**, 502-514
14. D'Souza, M.P., Ambudkar, S.V., August, J.T., and Maloney, P.C. (1987) Reconstitution of the lysosomal proton pump. *Proc. Natl. Acad. Sci. USA* **84**, 6980-6984
15. Bowman, E.J., Siebers, A., and Altendorf, K. (1988) Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA* **85**, 7972-7976
16. Kaunitz, J.D., Gunther, R.D., and Sachs, G. (1985) Characterization of an electrogenic ATP and chloride-dependent proton translocating pump from rat renal medulla. *J. Biol. Chem.* **260**, 11567-11573
17. Randall, S.K. and Sze, H. (1986) Properties of the partially purified tonoplast H^+-pumping ATPase from oat roots. *J. Biol. Chem.* **261**, 1364-1371
18. Moriyama, Y. and Nelson, N. (1989) Cold inactivation of vacuolar proton-ATPase. *J. Biol. Chem.* **264**, 3577-3582
19. Moriyama, Y. and Nelson, N. (1989) H^+-translocating ATPase in Golgi apparatus. Characterization as vacuolar H^+-ATPase and its subunit structures. *J. Biol. Chem.* **264**, 18445-18450
20. Bowman, B.J., Dschida, W.J., Harris, T., and Bowman, E.J. (1989) The vacuolar ATPase of *Neurospora crassa* contains an F_1-like structure. *J. Biol Chem.* **264**, 15606-15612
21. Moriyama, Y. and Nelson, N. (1987) Nucleotide binding sites and chemical modification of the chromaffin granule proton ATPase. *J. Biol. Chem.* **262**, 14723-14729
22. van Dyke, R.W. (1993) Acidification of rat liver lysosomes: Quantitation and comparison with endosomes. *Am. J. Physiol.* **265**, C901-C917
23. Dschida, W.J.A. and Bowman, B.J. (1995) The vacuolar ATPase: Sulfite stabilization and the mechanism of nitrate inactivation. *J. Biol. Chem.* **270**, 1557-1563
24. Sumner, J.-P., Dow, J.A.T., Earley, F.G.P., Klein, U., Jäger, D., and Wieczorek, H. (1995) Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem.* **270**, 5649-5653
25. Reenstra, W.W., Sabolic, I., Bae, H.-R., and Verkman, A.S. (1992) Protein kinase A dependent membrane protein phosphorylation and chloride conductance in endosomal vesicles from kidney cortex. *Biochemistry* **31**, 175-181
26. Xie, S.-X., Crider, B.P., and Stone, D.K. (1989) Isolation and reconstitution of the chloride transporter of clathrin-coated vesicles. *J. Biol. Chem.* **264**, 18870-18873
27. van Dyke, R.W. (1986) Anion inhibition of the proton pump in rat liver multivesicular bodies. *J. Biol. Chem.* **261**, 15941-15948
28. van Dyke, R.W. (1988) Proton pump-generated electrochemical gradients in rat liver multivesicular bodies: Quantitation and effects of chloride. *J. Biol. Chem.* **263**, 2603-2611
29. Fuchs, R., Schmid, S., and Mellman, I. (1989) A possible role for Na^+,K^+-ATPase in regulating ATP-dependent endosome acidification. *Proc. Natl. Acad. Sci. USA* **86**, 539-543
30. Corley-Cain, C., Sipe, D.M., and Murphy, R.F. (1989) Regulation of endocytic pH by the Na^+,K^+-ATPase in living cells. *Proc. Natl. Acad. Sci. USA* **86**, 544-548
31. Anbari, M., Root, K.V., and van Dyke, R.W. (1994) Role of Na, K-ATPase in regulating acidification of early rat liver endocytic vesicles. *Hepatology* **19**, 1034-1043
32. Fuchs, R., Mâle, P., and Mellman, I. (1989) Acidification and ion permeabilities of highly purified rat liver endosomes. *J. Biol. Chem.* **264**, 2212-2220
33. Hilden, S.A., Ghoshroy, K.B., and Madias, N.E. (1990) Na^+-H^+ exchange, but not Na^+-K^+-ATPase, is present in endosome-enriched microsomes from rabbit renal cortex. *Am. J. Physiol.* **258**, F1311-F1319
34. van Dyke, R.W., Root, K.V., Schreiber, J.H., and Wilson, J.M. (1992) Role of CFTR in lysosome acidification. *Biochem. Biophys. Res. Commun.* **184**, 300-305