Reconstitution of the lysosomal proton pump

(pH gradient/electrogenic pump/ion transport/octylglucoside/proteoliposome)

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ABSTRACT Lysosomal membrane proteins solubilized with octyl β-D-glucopyranoside were reconstituted into proteoliposomes using aceton/ether-washed phospholipids from Escherichia coli. Assays of the quenching of acridine orange fluorescence showed that addition of both ATP and valinomycin to K+-loaded proteoliposomes led to the formation of a pH gradient that was acidic inside. ATP-driven acidification took place in the absence of permeant anions and was inhibited by the "protonophore", carbonyl cyanide m-trifluoromethoxyphenylhydrazone, indicating that only H⁺ was transported actively. Proton translocation was readily blocked by N-ethylmaleimide (10 μM gave 50% inhibition of fluorescence quenching) but was unaffected by oligomycin (50 nM), orthovanadate (50 μM), or ouabain (0.5 mM); similarly, only N-ethylmaleimide inhibited ATP hydrolysis by proteoliposomes (88% inhibition). Other work showed that reconstitution of ATP-driven proton translocation required the presence of glycerol during protein solubilization and that optimal recovery depended on the use of both glycerol and phospholipid at this stage. We conclude that acidification of the lysosome is mediated by an ATPase capable of electrogenic H⁺ translocation without molecular coupling to other ionic species.

Lysozymes are the main components of a eukaryotic digestive system responsible for degradation of both extracellular and intracellular materials. The low internal pH of these organelles (about pH 5) provides an acidic environment suited to the operation of lysosomal hydrolytic enzymes (1–3). Studies of the isolated organelle indicate that its low internal pH is sustained by an ATP-driven H⁺ pump (4–9), which operates most effectively in the presence of permeant anions, in either an electrogenic (7, 8) or an electroneutral (9) fashion. This lysosomal H⁺ pump may be distinguished from other ion-motive pumps by its characteristic response to several inhibitors (7, 8). Lysosomal acidification is insensitive to both oligomycin and orthovanadate, inhibitors that block mitochondrial F₁F₀ and plasma membrane E₁E₂ solubilization ATPases, respectively, whereas levels of N-ethylmaleimide (MalNEt) that have no effect on either F₁F₀ or E₁E₂ pumps markedly reduce lysosomal acidification.

In its dependence on an external anion and in its pharmacological profile, the lysosomal H⁺ pump resembles ATP-driven acidification mechanisms associated with the eukaryote vacuolar membrane system—endoplasmic reticulum (10), Golgi membranes (11), vesicles mediating both endo- and exocytosis (12–17), and vacuoles (18–20). These minor organelles serve widely divergent functions, yet a superficial characterization suggests important similarities with respect to their resident H⁺ pumps. To determine whether this homology is retained at a more detailed level of analysis, we have examined the lysosomal H⁺ pump in a reconstituted state, with the specific goal of answering questions related to ionic selectivity.

MATERIALS AND METHODS

Preparation of Lysosomal Membranes. Liver lysosomes from NIH Swiss mice (20–25 g; Harlan Sprague Dawley, Indianapolis) were isolated using Triton flotation (21). To make membrane vesicles, lysosomes were swollen by 3-fold dilution into iced 10 mM 4-morpholinopropanesulfonic acid (Mops)/KOH (pH 7), 5 mM KCl, bovine serum albumin (5 mg/ml), 1 mM EDTA, and 1 mM diethiothreitol, containing protease inhibitors (2 mM p-aminobenzamidine/50 μM chymostatin/25 μM pepstatin/1 μM aprotonin/1 μM leupeptin). The mixture was incubated at 23°C with stirring for 15 min; 150 mM K₂SO₄ and 2.5 mM MgSO₄ were added; and after chilling, lysosomes were disrupted by brief hand homogenization (Dounce homogenizer). Unbroken material was removed by a low-speed centrifugation (20,000 × g; 10 min). Lysosomal membranes were collected by high-speed centrifugation (100,000 × g; 35 min), resuspended and washed in 250 mM sucrose, 20 mM Mops/KOH (pH 7), 1 mM diethiothreitol, 1 mM EDTA, and finally taken up in the same buffer containing protease inhibitors (see above) before storage at −70°C. Mitochondrial membranes were prepared in the same way using the mitochondrial fraction from the sucrose gradient [34%/45% (wt/wt) interface].

Solubilization and Reconstitution. The solubilization of membrane proteins followed the protocols established earlier (22), except that 1 mM EDTA and protease inhibitors were also present. Reconstitution by detergent dilution also followed a standard method (22); 0.5 mM EDTA was added to the loading buffer [150 mM K₂SO₄, 50 mM Mops/KOH, 1 mM diethiothreitol (pH 7)]. Proteoliposomes or liposomes were isolated by centrifugation, washed in 150 mM K₂SO₄, 50 mM Mops/KOH (pH 7), 2.5 mM MgSO₄, and taken up in 300 μl of the same buffer supplemented with protease inhibitors. Proteoliposomes contained 16–25% of the input membrane protein.

Assay of Proton Translocation. Formation of a pH gradient (interior acidic) was monitored by the quenching of fluorescence of acridine orange, a permeant weak base (23). The 2-ml assay medium (pH 7) contained 250 mM sucrose, 20 mM Mops/KOH, and 2.5 mM MgSO₄, as well as 50 μM of a liposome or proteoliposome suspension (0.7 mg of phospholipid; 2–25 μg of protein) and 6 μM acridine orange. In most cases the reaction was initiated by the addition of 1.25 mM K₂ ATP (bringing external potassium to 23 mM), followed by 0.5 μM valinomycin. Fluorescence was measured with an Aminco–Bowman spectrophotofluorometer, using excitation and emission wavelengths of 430 nm and 570 nm, respectively.

Abbreviations: DCCD, N,N'-dicyclohexylcarbodiimide; DIDS, 4,4'-disothiocyanato-2,2'-disulfonic acid; FCCP, carbonylcyanide m-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinopropanesulfonic acid; MalNEt, N-ethylmaleimide.
Other Methods. ATP hydrolysis was measured by release of $^{32}$P. Samples were incubated at 30°C with 2.5 mM [$\gamma$-$^{32}$P]ATP (0.8 Ci/mmol; 1 Ci = 37 GBq) in 2 ml of 150 mM KCl, 20 mM Mops/KOH (pH 7), and 5 mM MgSO$_4$. At intervals, 200-μl aliquots were mixed with 100 μl of 30% trichloroacetic acid, and after centrifugation, $^{32}$P, was extracted from the supernatants as the molybdate complex (24). Specific antibody binding was estimated by a solid-phase assay (25), and standard techniques were used in assays of β-N-acetylgalactosidase (26) and membrane protein (22, 27).

Chemicals. Asolectin and Escherichia coli phospholipids were purified (22, 28) from crude mixtures provided by Avanti Polar Lipids. Avanti also supplied purified phosphatidycholine (bovine liver) and phosphatidylserine (bovine brain). [$\gamma$-$^{32}$P]ATP (3000 Ci/mmole) and carrier-free Na$^{125}$I were from Amersham. Jackson Immunoresearch (Avondale, PA) was the source of reagents for the immunological tests. All other chemicals were from Calbiochem–Behring or Sigma.

RESULTS

Immunological Studies. It is known that lysosomes prepared by Triton flotation are free of mitochondrial, peroxisomal, and microsomal enzymes (21), and to supplement this biochemical description, we used immunological tests with antibodies directed against markers of known location. A monoclonal antibody reactive to a plasma membrane antigen, Ly-24 [Pgp-1 (29)], gave no detectable binding to proteoliposomes, whereas monoclonal antibodies specific for the lysosome-associated membrane proteins LAMP-1 and LAMP-2 (30) were bound at levels 25- to 50-fold greater than those found in the primary homogenate (Table 1). Similar assays showed that neither lysosomes nor proteoliposomes reacted with a polyclonal serum raised against the β subunit of rat liver mitochondrial F$_1$, even though this serum was reactive with various liver mitochondrial fractions.

Although immunological studies excluded a significant contamination of lysosomal preparations by either mitochondrial or plasma membranes, such tests did suggest the presence of endosomes or other vesicular material, since a monoclonal antibody against the putative α$_2$-macroglobulin receptor (31) was increased 5-fold in both lysosomal and proteoliposomal fractions (Table 1). Endosomal contamination was also indicated by recovery, in the lysosomal fraction, of a freshly administered endosomal marker, $^{125}$I-labeled asialoorosomucoid (32) (not shown). In the absence of biochemical markers for endosomes and other low-density organelles, the extent of contamination by such components was estimated by the direct comparison of material obtained from untreated mice or mice treated with Triton WR-1339. When mice had not received Triton, protein recovered at the 14%/34% (wt/wt) interface (lysosomal fraction) was reduced to 17% of the normal yield. Of this material, about one-third was judged to be lysosomal [presumably low-density lysosomes (26)], because the specific activity of β-N-acetylgalactosidase decreased from 100,000 to 30% of 100 μl of 30% trichloroacetic acid, and after centrifugation, $^{32}$P, was extracted from the supernatants as the molybdate complex (24). Specific antibody binding was estimated by a solid-phase assay (25), and standard techniques were used in assays of β-N-acetylgalactosidase (26) and membrane protein (22, 27).

Table 1. Immunological profile of mitochondria, lysosomes, and proteoliposomes

<table>
<thead>
<tr>
<th>Test antibody</th>
<th>Primary homogenate</th>
<th>Mitochondria</th>
<th>Lysosomes</th>
<th>Proteoliposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ly-24</td>
<td>1.0</td>
<td>0.2</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-LAMP-1</td>
<td>1.3</td>
<td>11.3</td>
<td>30.3</td>
<td>33.9 (22.2)</td>
</tr>
<tr>
<td>Anti-LAMP-2</td>
<td>1.9</td>
<td>15.0</td>
<td>36.9</td>
<td>45.4 (23.9)</td>
</tr>
<tr>
<td>Anti-α$_2$-macroglobulin receptor</td>
<td>2.3</td>
<td>2.4</td>
<td>13.1 (8.3)</td>
<td>12.6 (10.0)</td>
</tr>
<tr>
<td>Anti-F$_1$ (rat liver)</td>
<td>0.8</td>
<td>5.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results from four separate experiments are shown. Specific binding reflects $^{125}$I-labeled anti-rat or anti-rabbit immunoglobulin antibody bound after subtracting background (300–800 cpm). For samples of the primary liver homogenate, mitochondria, or lysosomes, assays with 1 μg of protein per well gave conditions of antibody excess. To achieve antibody excess when proteoliposomes were tested, the antigen was used at 0.2 μg per well (data in parentheses). Proteoliposomes were made with lysosomal membrane protein. ND, specific binding was not detectable; LAMP-1 and LAMP-2, lysosome-associated membrane proteins.
pH gradient, whereas prior use of FCCP prevented the reaction altogether (Fig. 1B). Indeed, this pattern of response to H⁺- and K⁺-selective ionophores both documented ATP-driven H⁺ movements and excluded the operation of either a K⁺-motive ATPase or an ATP-linked nH⁺/K⁺ exchange (33).

Three observations have ruled out the possibility that this electrogenic proton translocation (Fig. 1B) reflected the unsuspected presence of mitochondrial F₁₀F₁. First, immunological and enzymatic tests were negative for mitochondrial contamination (Table 1). Second, ATP-driven acidification reconstituted from lysosomal membranes was inhibited by MalNEt but not by oligomycin (Fig. 1B). Third, and most important, when mitochondrial F₁₀F₁ was itself reconstituted, ATP-driven H⁺ transport responded to valinomycin and FCCP as before, but the reactivity to MalNEt and oligomycin was reversed (Fig. 1C).

A further pharmacological description of the lysosomal H⁺ pump was provided by the experiments outlined in Fig. 2. There were, for example, no significant effects of oligomycin (50 nM), orthovanadate (50 μM), ouabain (0.5 mM), or NaN₃ (1 mM; not shown), but 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 50 μM), N,N',dicyclohexyl carbodi-imide (DCCD; 100 μM), and MalNEt (1 mM) gave complete inhibition. Since DIDS and DCCD also block mitochondrial F₁₀F₁ (34), the selective effect of MalNEt was examined more carefully. That work showed half-maximal inhibition of fluorescence quenching by 10 μM MalNEt (Fig. 2B).

Fig. 1. Reconstitution of lysosomal and mitochondrial proton-translocating ATPases. Each panel records the fluorescence of acridine orange in suspensions of K⁺-loaded liposomes or proteoliposomes. (A) Liposomes (10 μl) washed free of external K⁺ were suspended in 2 ml of 250 mM sucrose, 20 mM Mops/choline (pH 7), and 2.5 mM MgSO₄ before adding 0.5 μM nigericin (trace 1) or 0.5 μM valinomycin (trace 2; arrowhead). Additions of 1.5 mM K₂SO₄ (to 24 mM K⁺) are indicated by open arrows; for valinomycin-treated liposomes, the final level of fluorescence in the presence of 24 mM K⁺ is given by the dashed line. (B) In a separate experiment, 50 μl of proteoliposomes (14.7 μg of lysosomal membrane protein; traces 1–3 and 5) or liposomes (trace 4) were suspended in 250 mM sucrose, 20 mM Mops/KOH (pH 7), and 2.5 mM MgSO₄ along with either ethanol (trace 1), 50 nM oligomycin in ethanol (trace 2), or 1 mM MalNEt (trace 3) before adding K₂ ATP (downward arrow) and valinomycin (arrowheads). 1 μM FCCP was added first in trace 5 but last in the other cases (upward arrows; traces 1–3). (C) Conditions were as in B, except that the proteoliposomes (50 μl, 23.1 μg of protein) contained mitochondrial protein. For trace 1 (ethanol control), trace 2 (1 mM MalNEt), and trace 3 (50 nM oligomycin), additions were in the order ATP, valinomycin (arrowheads), and FCCP (upward arrows).

Fig. 2. Inhibition of the lysosomal proton-translocating ATPase. (A) Proteoliposomes (5.5 μg of protein) were assayed as in Fig. 1B with the following test agents: ethanol control (trace 1); 50 nM oligomycin (trace 2); 0.5 mM ouabain (trace 3); 50 μM orthovanadate (trace 4); 50 μM DIDS (trace 5); 100 μM DCCD (trace 6). The order of additions was ATP, valinomycin (arrowheads), and then 0.5 μM nigericin (arrows). (B) In a separate experiment, proteoliposomes (4.2 μg of protein) were suspended as A and treated with MalNEt for 3 to 5 min at 23°C before adding ATP and valinomycin (arrowheads); the reaction was terminated by nigericin (arrows). MalNEt was used at 0 μM (trace 1), 10 μM (trace 2), 25 μM (trace 3), 50 μM (trace 4), and 125 μM (trace 5).
Phospholipid Requirements. Reconstitution of the lysosomal H\(^+\) pump has exploited methods developed to study bacterial transport proteins (22, 35, 36). In those instances, success had required the presence of stabilizing agents (glycerol and phospholipid) at the time of protein solubilization (22), and it was of practical significance to evaluate these parameters in the present work (Fig. 3). Such trials have demonstrated that the use of glycerol and an osmolyte stabilizer (22, 37), was essential for reconstitution of ATP-driven acidification (Fig. 3A) and that although dependence on phospholipid was less strict, optimal recovery was not achieved unless both glycerol and lipid were present during solubilization (Fig. 3A). Perhaps most interesting, among the lipids examined as vehicles for reconstitution, there was an apparent preference for those derived from E. coli, despite the fact that the bacterial mixture lacks phosphatidylcholine, phosphatidylglycerol, sphingomyelin, and cholesterol, which together comprise about 80% of lysosomal membrane lipids (38). By contrast, ATP-driven acidification was not recovered when solubilization and reconstitution used purified phosphatidylcholine and phosphatidylserine (50:50, wt/wt), and only a partial success was recorded with asolectin (Fig. 3B).

Reconstitution of ATPase Activity. To correlate reconstitution of ATP-driven H\(^+\) transport with other biochemical parameters, we also studied the recovery of ATPase activity (Fig. 4). Native lysosomal membranes had a total ATPase activity somewhat higher than that of derived proteoliposomes (means of 1.55 and 0.87 \(\mu\)mol per mg per min of protein, respectively), but, due to differential inhibition by MalNEt (44% ± 6% and 88% ± 4%, respectively; means ± SEM of four experiments), the two preparations had about the same MalNEt-sensitive ATPase component (0.68 and 0.77 \(\mu\)mol per min per mg of protein, respectively). Such work showed also that ATP hydrolysis by lysosomes and proteoliposomes was unaffected by oligomycin (50 nM) (Fig. 4) or orthovanadate (50 \(\mu\)M) (not shown).

**DISCUSSION**

The experiments described here report the reconstitution of an ATP-driven acidification mechanism derived from lysosomes. In the artificial system, application of both immunological (Table 1 and text) and pharmacological tests (Figs. 1 and 2) has confirmed the placement of this pump within the family of proton-translocating ATPases now recognized as being associated with the internal vacuolar system of the eukaryote cell (reviewed in refs. 12, 20, and 39). Definition of this additional family of ion-motive ATPases has relied mainly on tests of intact organelles, and such studies generally find that organelle acidification requires both ATPase function and a suitable external anion. The need for a permeant external anion (usually chloride) is often attributed to the operation of a separate channel or carrier that allows electrophoretic anion entry (8, 11, 40), but without corroborating tests in an artificial system, the evidence remains compatible with a coupling between proton and anion movements at the level of the ATPase itself (e.g., ref. 41). For the lysosomal H\(^+\) pump, the present work now offers an unambiguous distinction between these alternatives, since the reconstituted enzyme functioned in the absence of permanent anions as long as internal K\(^+\) and valinomycin were present (Fig. 1B). For this reason and because the responses to selective ionophores ruled out a direct participation of K\(^+\), we conclude that the lysosomal ATPase mediates an electrogenic H\(^+\) translocation without a molecular coupling to other ionic species. The same conclusion has been reached for the reconstituted H\(^+\) pump of the vacuolar lysosome (42, 43). It will be of interest if continued analysis in artificial systems verifies thisionic selectivity for other examples of the vacuolar H\(^+\) pump.

The vacuolar and FoF1 ATPases each catalyze an electrogenic H\(^+\) translocation (e.g., Fig. 1 B and C). Moreover, recent biochemical studies suggest structural similarities between the vacuolar and FoF1 ATPases in regard to overall molecular mass (18-20, 42), the presence of a pair of nucleotide binding subunits (18, 44), and the participation of a small DCDC-reactive subunit (17-19, 44-46). These observations support the idea that these two families belong to a larger class of H\(^+\) pumps sharing a fundamental mechanistic homology and a common evolutionary origin (39).

Certain of the experiments described here have general implications for the interpretation of protein–lipid interactions by the vacuolar family of H\(^+\) pumps. In particular, it appears that the lysosomal example can operate in an environment dominated by bacterial phospholipid. Clearly, the MalNEt-sensitive ATPase activity of native membranes was recovered intact (Fig. 4 and text), and an indirect argument based on the assay of H\(^+\) transport suggests that the reconstituted enzyme shows a high turnover number. In

![Fig. 3. Effects of glycerol and phospholipid on reconstitution of the lysosomal H\(^+\) pump. (A) Assay conditions were as described in Fig. 2A for control proteoliposomes (4.2 \(\mu\)g of protein) (trace 1) and for proteoliposomes (2.5 \(\mu\)g of protein) made without phospholipid (trace 2) or without glycerol (trace 3) during solubilization. (B) Proteoliposomes (2.1-2.5 \(\mu\)g of protein) were tested as described in Fig. 2A. Solubilization and reconstitution of lysosomal protein used either E. coli phospholipid (trace 1), the same quantity of asolectin (trace 2), a 50:50 mixture of phosphatidylcholine and phosphatidylserine (trace 3), or a 20:40:40 (wt/wt/wt) mixture of cholesterol, phosphatidylcholine, and phosphatidylserine (trace 4). Nigericin-induced fluorescence quenching was found when these same proteoliposomes were diluted 160-fold into a choline-based buffer (cf. Fig. 1A).](image)

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**Fig. 4.** ATP hydrolysis by lysosomal membranes and proteoliposomes. ATPase activity was assayed, using either lysosomal membranes or proteoliposomes with protein derived from these same membranes. Samples were pretreated for 5 min at 23°C with ethanol ( ), 50 nM oligomycin ( ), or 1 mM MalNEt ( ). (A) Lysosomal membranes (11.6 \(\mu\)g of protein). (B) Proteoliposomes (6 \(\mu\)g of protein).
the experiment of Fig. 1, for example, reconstitution with mitochondrial membrane protein (35 μg of protein per mg of phospholipid) should have yielded \( \approx 3 \) FeF₄ per proteoliposome, since this 560-kDa enzyme represents about 10% of the mitochondrial membrane protein (47) and since reconstitution by octyl glucoside dilution yields about 2 \( \times \) 10⁵ particles per mg of phospholipid (22, 48). Given that the lysosomal and mitochondrial enzymes establish the same pH gradient, the different responses (36% and 79% fluorescence quenching, respectively; Fig. 1 B and C) should reflect only different numbers of active enzymes. In turn, if acridine orange, a bifunctional amine, behaves as a weak base (23), the simplest model accommodating the fluorescence data requires that the lysosomal pump was present in one-seventh as many proteoliposomes as FeF₄. For the experiment in question, then, the lysosomal pump would have been present at 0.29 \( \times \) 10⁵ enzymes per mg of phospholipid, or 23 pmol per mg of protein. Together with the measurement of MalNIT-sensitive ATPase (0.57 μmol per min per mg of protein), this calculation predicts a turnover number of 420/sec with respect to ATP. Because this value is in the expected range (47, 49), we conclude that the lysosomal pump was reconstituted as an active entity.

The phospholipids of lysosomal membranes are largely phosphatidylincholine (41% by weight), phosphatidylethanolamine (26%), phosphatidylserine (10%), phosphatidylinositol (8%), and cholesterol (at a molar ratio of cholesterol to total phospholipid of 0.3:1) (38). By contrast, the acetone/ether-washed material from *E. coli* lacks cholesterol and has only phosphatidylethanolamine (70%), phosphatidylglycerol (15%), and cardiolipin (15%) (48). If lysosomal and bacterial lipids had mixed randomly during reconstitution, one would expect proteoliposomes to have <2% of their phospholipids derived from the lysosomal source. Since phosphatidylethanolamine is the only lipid common to both sources, we conclude that the lipid specificity (if any) of the lysosomal H⁺ pump is restricted to this single species. This finding is of interest in view of a complex lipid requirement ascribed to the H⁺ pump of clathrin-coated vesicles (43). These differences may arise because of different tactics used to preserve enzymatic activity during solubilization and reconstitution, and, if so, our findings have practical value in their demonstration that inactivation accompanying solubilization may be avoided by use of an osmolyte stabilizer, glycerol (22). A lipid dependence during reconstitution of the clathrin H⁺ (43) may also indicate regulation exerted by membrane lipid, and in this case our results would document a fundamental difference between the lysosome and clathrin pumps, since the lysosomal enzyme mediates efficient H⁺ transport and ATP hydrolysis in the absence (<2%) of native lipid.

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