Solubilization and Reconstitution of the Gastric H,K-ATPase*

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Proteoliposomes containing the hog gastric H⁺,K⁺-ATPase were prepared from cholate and n-octyl glucoside extracts of native microsomes. Experiments were presented which show reconstitution-dependent selective purification of a 94-kDa peptide capable of Rb⁺/Rb⁺ exchange and active H⁺ transport. The absence of selective enrichment of residual protein contamination in this material suggests but does not prove that those transport reactions are attributable only to the 94-kDa peptide. Transport demonstrated inhibitor sensitivity and cation specificity comparable to the accounted occluded enzyme form in the H,K-ATPase. A model passive exchange and active Rb⁺ efflux suggest that those transport reactions are attributable only to the selective purification of a 94-kDa peptide capable of Rb⁺/Rb⁺ exchange and active H⁺ transport. The absence of selective enrichment of residual protein contamination in this material suggests but does not prove that those transport reactions are attributable only to the 94-kDa peptide. Transport demonstrated inhibitor sensitivity and cation specificity comparable to the occluded state in the catalytic or transport reactions (9, 16). In gastric microsomes, a Rb⁺/Rb⁺ exchange pathway of a magnitude similar to active Rb⁺ transport was demonstrated (17, 18). In terms of purification, the highest catalytic activity has been obtained from vesicles obtained by a combination of centrifugation and free flow electrophoretic techniques. Although there appears to be a single molecular weight species responsible for enzyme activity, some doubt exists as to its homogeneity due to separation of peptides according to isoelectric point (19).

Recently, the gastric ATPase has been solubilized by n-octyl glucoside (20), and in this paper it is demonstrated that a transporting vesicle of higher specific activity and simpler peptide pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis than observed previously is produced by reconstitution of the n-octyl glucoside-solubilized ATPase. This manuscript demonstrates that it is possible to solubilize and reconstitute the gastric ATPase from various detergents with a variety of lipids and that these reconstituted systems can be used to determine the minimal peptide composition of the transporting enzyme, its electrogenicity, and the relationships of K⁺ transport to the catalytic peptide.

EXPERIMENTAL PROCEDURES

Preparation of Gastric Microsomes

Gradient fractionated microsomes were used as the source material for both cholate and n-octyl glucoside-induced membrane dissociation. This material was obtained by methods previously described (4, 13). Briefly, the hog gastric fundus was scraped, homogenized in a glass homogenizer with Teflon pestle set at 2300 rpm, and a microsomal pellet obtained by differential centrifugation. Purified gastric microsomes were then obtained by sedimentation through Ficoll/sucrose gradients. Either the Beckman Z-60 or Sorvall TZ-28 zonal rotors were used for this purpose. In the latter case, microsomes at an approximate protein concentration of 5 mg/ml in 0.125 M sucrose and 5 mM Pipes-Tris, pH 6.8, were applied to a step gradient consisting of 650 ml of 0.25 M sucrose, 240 ml of 7% Ficoll (w/w) in 0.25 M sucrose, 350 ml of 34% sucrose (w/w), and 100 ml of 60% sucrose, all with buffer as above at pH 6.8. With both rotors, the material collected at the 0.25 M sucrose/Ficoll and the Ficoll/34% sucrose interface was diluted 1:3, washed once with 5 mM Pipes-Tris, pH 6.8 buffer, resuspended to a protein concentration of 5.5 mg/ml, and stored at -80 °C until use.

Liposome Preparation

Liposomes were prepared, in general, as described by Kasahara and Hinkle (21). One hundred mg of lipid were dissolved in chloroform and added to 15-ml borosilicate tubes. The solvent was evaporated

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Detergent Solubilization and Reconstitution

In general, for cholate-dependent membrane dissociation, gastric microsomes were suspended at a concentration of 1–2 mg/ml protein in a medium containing 5 mM Pipes/Tris, pH 6.1, and either 75 mM K_2SO_4 or 0.25 M sucrose. In preliminary experiments, cholate was added over a concentration range from 0.3 to 2%, the mixture vortexed for 10 s, and then incubated for 5 min on ice. For reconstitution of the supernatant enzyme, the suspension was then centrifuged for 30–60 min at 10^5 × g, and the upper 80% of the cleared suspension carefully decanted before mixing with a liposomal suspension at 3–12 °C. The detergent was removed by the disassociated membranes were mixed directly with liposomes. It was noted that increasing centrifugation time from 30 min to 2 h did not increase the yield of pelleted protein. Solubilized enzyme was functionally defined as that protein released into the 10^5 × g supernatant following detergent treatment. Successful reconstitution was achieved with both the original detergent-treated suspension and the supernatant following centrifugation. The phospholipid/detergent/protein ratios were usually adjusted to stand for 5 min on ice and sonicated for times ranging from 30–240 s. The optimum sonication time was determined empirically for each reconstitution, and in some cases it was useful to incorporate a freeze/thaw (dry ice/methanol) cycle in the procedure prior to the sonication step. In the experiments reported, 1.5% cholate was utilized for solubilization with a 2-fold dilution during reconstitution. It was found that H^+ transport activity of proteoliposomes could be maintained for at least 1 month by storage at -80 °C in 20% glycerol.

Gastric microsomes solubilized by n-octyl glucoside were suspended at 4–5 mg/ml protein in a medium consisting of 20 mM Mes/Tris pH 5.5, 1 mM MgATP, and 0.25 M sucrose. n-Octyl glucoside (25%) was added to a final concentration of 2.5%. This mixture was then incubated for about 5 min at 4 °C and diluted 4–16-fold in MgATP-free buffer containing a supernatant obtained or incubated for 1 min and mixed immediately with liposomes (60% phosphatidylcholine, 40% cholesterol at 15 mg/ml). In the latter case, transport functions were reconstituted by detergent dilution, following centrifugation through a sucrose density gradient, dilution (60-fold) into assay medium, or filtration through Sephadex G-50.

Assay Procedures

Measurement of H^+ Transport—MgATP-dependent H^+ transport was measured in proteoliposomes by the fluorescent quench of acridine orange (22). H^+ transport activity was measured at room temperature using either a Perkin-Elmer MFP 44 or 650/40 fluorometer. In a typical experiment, 50 µl of the reconstituted material (~15–50 µg of protein) was added to 2 ml of the reaction medium which contained as necessary, K^+ or TMA^+, CCl_4 or SO_4^2- at concentrations as specified in the text, and 5 mM Pipes-Tris buffer at either pH 6.1 or 7.4.

Measurement of Potential—Potential measurements utilized the absorbance change of the cationic potential probe, DOCC, by methods detailed previously (23). The ATP-dependent proton generation was determined at 4 °C and at a water level of 75 mM K_2SO_4, which was diluted into 2.0 ml of a similar ionic medium containing 5.0 mM TCS. H^+ transport was initiated by addition of 0.5 mM MgATP.

Measurement of Rb^+ Flux—Rb^+ uptake was measured in the presence of 0.1 to 20 mM extravesicular Rb^+ by the Dowex procedure (17, 24). In a typical experiment, proteoliposomes at approximately 1 mg/ml protein, 6 mg/ml lipid, 75 mM Rb_2SO_4, and 10 mM Pipes/Tris, pH 6.1, were filtered twice on Sephadex G-50 equilibrated in 75 mM tetramethyl ammonium sulfate and 10 mM Pipes/Tris, pH 6.1, to remove extravesicular Rb^. Rb^+ uptake was initiated by addition of 100-µl aliquots of the proteoliposomes over Dowex 50-W equilibrated in 0.25 M sucrose. The column was washed with two 0.5-ml volumes of 0.25 M sucrose. Inhibitors were added to proteoliposomes in 0.03 M Pipes at pH 6.1. After sonication, the suspensions were stored at 4 °C under N_2 for up to 5 days. The suspended liposomes were sonicated at an additional 15 min each day prior to use in reconstitution studies.

Materials

All chemicals were the highest grade available. Lipids were obtained as noted. Labeled ATP was obtained from ICN and 86Rb from Amersham Corp. Cholate was obtained from Sigma and recrystallized before use. Omeprazole was obtained from Hassle, Molndal, Sweden.

RESULTS

Solubilization of the H,K-ATPase—An n-octyl glucoside extraction of the gastric ATPase has been previously described (20). In this investigation both this detergent and cholate have proved useful for reconstitution of gastric ATPase transport functions. In general, the former detergent was most useful for purification of the enzyme while the latter was well suited for ion transport studies.

Membrane-bound proteins were extracted with both detergents, though with n-octyl glucoside recovery of supernatant protein was less. With n-octyl glucoside, 20–40% of the total proteoliposomes could be pelleted following detergent extraction with 2.4% detergent. The activity of this diluted supernatant enzyme was about 40% of the specific activity of the original K^+-stimulated ATPase. In contrast, the yield of solubilized protein recovered as supernatant enzyme was greater with cholate treatment (56% at 1.5% cholate), though recovery of ATPase activity was significantly less. The supernatant enzyme activity in cholate-extracted microsomes was typically less than 10% of native activity. However, detergent-extracted gastric microsomes prior to sedimentation maintained approximately 25% of the original ATPase activity. As will be shown below, ATPase activity as well as the partial reactions of the ATPase, i.e. p-nitrophenylphosphatase and K^+—sensitive phosphoenzyme, can be recovered in reconstituted proteoliposomes.

Liposomal Requirements for Reconstitution of H^+ Transport—Though endogenously associated lipids were undoubtedly present in these detergent extracts, H^+ transport capacity was lost in both supernatant and pelleted protein fractions following detergent treatment. Reconstitution of H^+ transport was present in the supernatant and pelleted protein fractions following detergent treatment with both the quantity and composition of liposomes utilized for the reconstitution.

Initial experiments were performed with cholate-extracted supernatant enzyme. It was subsequently learned that transport activities reconstituted from the supernatant fraction could also be reconstituted from the detergent-treated extract without separation of the supernatant enzyme. This procedure was especially useful with n-octyl glucoside-extracted enzyme because rapid addition of phospholipids following detergent treatment prevented irreversible loss of enzyme function.

The lipid composition of the liposomes affected both the frequency of successful reconstitution as well as the optimum reconstituted transport activity. H^+ transport activity was most reproducibly reconstituted in liposomes composed of equal mixtures of cholesterol and phospholipid (w/w). In cholate-treated material, independent of the lipid mixture of
the added liposomes, optimum transport was observed at 6–9 mg of lipid/mg of protein. A similar lipid dependence was noted following reconstitution of n-octyl glucoside-extracted membranes. In the latter case, H⁺ transport activity was optimum at a lipid/protein ratio of about 5 (15 mg ml⁻¹ lipids and 3 mg ml⁻¹ protein). In subsequent experiments, liposomes composed of phosphatidylcholine:cholesterol (60:40) were utilized for reconstitution of H⁺ transport activity following membrane extraction with either cholate or n-octyl glucoside.

H⁺ Transport—In preliminary experiments with reconstituted cholate extracts it was determined that simple addition of KCl or K₂SO₄ to the external medium did not support protonophore sensitivity than those reconstituted in a parallel transport. In proteoliposomes reconstituted from either cholate- or n-octyl glucoside-extracted enzyme, the cation requirement for H⁺ transport exhibited both sidedness and specificity. Substitution of various monovalent cations (cation入户 = cationout) for K⁺ showed that only Rb⁺ substitution for K⁺ supported H⁺ transport. In proteoliposomes reconstituted from either cholate- or n-octyl glucoside-extracted enzyme, the % for H⁺ transport was on the order of 1–3 min at room temperature, in contrast to 10–20 s described for native vesicles under similar conditions (26).

As seen in Fig. 1, maximal acidification was obtained in K₂SO₄, rather than KCl-loaded proteoliposomes. In the latter, the decreased pH gradient was attributed to a leak of HCl. Evidence for this was shown in Fig. 2 where proteoliposomes reconstituted in KCl showed significantly greater protonophore sensitivity than those reconstituted in a parallel experiment in K₂SO₄. Additionally, in these reconstituted proteoliposomes the K⁺ conductance was lower than the Cl⁻ conductance, since the HCl leak in KCl-loaded proteoliposomes but not H⁺/K⁺ exchange in K₂SO₄-loaded proteoliposome exceeded the pump capacity in the presence of TCS. As mentioned above, a MgATP-dependent pH gradient was not found following dilution of K⁺-free proteoliposomes into KCl media. Finally, since the H⁺ gradient was relatively insensitive to TCS in K₂SO₄ media, the H⁺ pump was not directly electrogenic since the TCS-induced H⁺ conductance would then uncouple the pump.

Electroneutrality was further substantiated in this series of reconstituted cholate extracts by the use of potential sensitive dye probes. In Fig. 3, it was shown that in the absence of TCS, the cationic probe DOCC was unresponsive to addition of MgATP. However, upon addition of TCS to the K₂SO₄-loaded liposomes, a nigericin-sensitive dye response was observed, as predicted for an outward H⁺ diffusion potential.

Inhibition of the rate of H⁺ transport was observed at decreased extravesicular K⁺. As shown in Fig. 4, in the reconstituted cholate system the Kₑ₆ for inhibition was about 40 mM at pH 7.4 and 1.0 mM MgATP. The inhibition was pH dependent, being significantly less at pH 6.1.

This reconstituted H⁺ transport was sensitive to inhibitors of enzyme activity (Table I). Both H⁺ transport and K⁺-stimulated ATPase were inhibited by vanadate, a potent inhibitor of this class of ATPase, and omeprazole, a substituted benzimidazole inhibitory to the gastric ATPase (27). Slight oligomycin sensitivity was also observed.

Cation Transport—Active transport by the H,K-ATPase occurs by exchange of cytoplasmic H⁺ for luminal K⁺. Native vesicles have been shown to actively extrude Rb⁺ from equilibrium (Rb⁺ₑ = Rb⁺ₕ) following MgATP addition, even in the presence of a protonophore (30). The active component of Rb⁺ flux in the n-octyl glucoside-reconstituted system is shown in Fig. 5. In this experiment approximately 80% of the Rb⁺ content of the proteoliposomes (Rb⁺ₑ = Rb⁺ₕ = 4.0 mM) was rapidly extruded following addition of MgATP. In parallel
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**TABLE I**

**Inhibitor correlation of ATPase and H⁺ transport**

For inhibitors of the reconstituted H,K-ATPase activity is reported as per cent of control at each indicated inhibitor concentration. Proteoliposomes reconstituted from cholate were resolved on sucrose gradients, prior to assay. Activity was measured from aliquots recovered from the peak of reconstituted H⁺ transport activity, and ATPase activity, measured in the presence of Mg⁺ and K⁺, were determined following a 10-min incubation of the inhibitor and the sample. Omeprazole was activated prior to its addition to the assay media by a 2-min incubation at pH 2.0.

| Inhibitor | Cholate | H⁺ transport | ATPase activity |
|-----------|---------|--------------|-----------------|
| NaN₃VO₄   | H⁺      | 63           | 80              |
| 10 μM     |         | 35           | 43              |
| 30 μM     |         | 10           | 36              |
| 70 μM     |         | 80           | 100             |
| Omeprazole| 30 μM   | 52           | 34              |
| 100 μM    |         | 6            | 0               |
| Oligomycin| 1 μg/ml | 73           | 88              |
| 10 μg/ml  |         | 50           | 76              |
| 15 μg/ml  |         | 43           | 66              |

**FIG. 3.** H⁺ pump potential due to the reconstituted H,K-ATPase. Lipophilic cation uptake (DOCC) was measured following addition of 0.5 mM MgATP to the reconstituted ATPase suspended in an assay buffer containing 75 mM K₂SO₄, 130 mM sucrose, 5 mM Pipes/Tris, pH 6.1, and 4 μM DOCC ± 5 μM TCS. At the indicated time the TCS-dependent diffusion potential was dissipated by addition of 5 mM 1,2-(cyclohexylenedinitrilo)tetraacetic acid (CDTA). The absorbance change in the absence of TCS was insensitive to nigericin.

**FIG. 4.** Inhibition of H⁺ uptake by cytoplasmic K⁺. Proteoliposomes reconstituted from cholate extracts containing 75 mM K₂SO₄ were diluted in transport buffer containing isotonic tetramethyl ammonium sulfate/K₂SO₄ combinations, 0.125 M sucrose, 5.0 mM Pipes/Tris, pH 7.4, and 1 μM acridine orange. The maximal rate of H⁺ transport was determined following addition of 1 mM MgATP.

The relatively rapid approach to isotopic equilibrium observed for H⁺ uptake under conditions similar to Fig. 5 (prior to substrate addition) suggested that the proteoliposomes, like native vesicles, possess a cation/cation exchange mechanism. This passive exchange, shown in Fig. 6, was resolved into 2 components, one saturable and the other nonsaturable. The former was identified as Mg⁺ and vanadate sensitive and accounted for approximately 90% of H⁺ uptake at the measured of 1.5 mM Rb⁺. Vanadate was used to determine the symmetry of protein incorporation into the lipid bilayer. The experiment in Fig. 7 showed that vanadate inhibition of the saturable exchange was dependent on the side at which vanadate was added. The addition of extravesicular vanadate inhibited about 70% of the saturable Rb⁺ flux. An additional 30% of the Rb⁺ flux was insensitive to extravesicular vanadate but sensitive to internal vanadate. Omeprazole, a relatively selective inhibitor of the H,K-ATPase also blocked the saturable component. A
The experiment shown in Table I was designed to determine whether the total =Rb⁺ uptake; 0, vanadate-sensitive =Rb⁺ uptake; Δ, vanadate-insensitive =Rb⁺ uptake. The experiment shown in Fig. 6 suggested that a Rb⁺/cation exchange was present with a selectivity of K⁺ ≈ Rb⁺ > Na⁺ > Li⁺ > TMA⁺. The H⁺ transport activity exhibited the same order of cation selectivity following dilution of the cation-loaded proteoliposomes into Rb₂SO₄ medium. These results suggested that the intravesicular K⁺ requirement for H⁺ transport was met by Rb⁺ or K⁺ uptake through passive K⁺/cation exchange, prior to the addition of MgATP.

Though enzyme kinetic data (28) have suggested an interaction between the binding of protons and potassium in the phosphorylated enzyme this relationship has not been examined in the unphosphorylated cation exchanger. As discussed below, it is likely that the saturable cation exchange is due to cation transport through alternating states of the catalytic peptide(s). Vanadate sensitivity, as observed, would be predicted if this is so. In addition, dependent upon the existence of a ligand-sensitive rate-limiting K⁺ occluded state (9), differential effects of nucleotide or Mg phosphate could be predicted. As shown in Table III, in the absence of Mg²⁺, ATP inhibition of cation exchange was observed at all cytoplasmic Rb⁺ concentrations within the range of 0.2 and 2 times the K₉₅ for this cation. At 1.0 mM ATP, this inhibition accounted for about 60 and 80% of vanadate-sensitive cation exchange at 0.3 and 3.0 mM Rb⁺, respectively. ATP-dependent stimulation of cation exchange was not observed. The influence of Mg²⁺ and phosphate is shown in Table IV. In contrast to ATP, up to 2-fold stimulation of exchange was induced by 5 mM phosphate and low Mg²⁺. At high Mg²⁺ concentrations, neither stimulation nor inhibition of exchange by phosphate was observed.

**Purification of the Reconstituted H,K-ATPase**—Presumably
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TABLE IV
Influence of Mg²⁺ P_i on "Rb⁺ exchange

| Exchange | 0.1 mM Mg²⁺ | 3.0 mM Mg²⁺ |
|----------|------------|------------|
| Rb⁺, = 0.1 mM |           |            |
| Control   | 120        | 107        |
| 0.3 mM P_i| 76         | 111        |
| 1.0 mM P_i| 105        | 120        |
| 5.0 mM P_i| 193        | 108        |
| Rb⁺, = 1.0 mM |           |            |
| Control   | 430        | 506        |
| 0.3 mM P_i| 605        | 645        |
| 1.0 mM P_i| 746        | 419        |
| 5.0 mM P_i| 1008       | 589        |

FIG. 8. Influence of pH on kinetic parameters of Rb⁺ exchange. After reconstitution of cholate extracts, the vanadate-sensitive component of "Rb⁺ uptake into proteoliposomes containing 75 mM Rb₂SO₄ was measured over the pH range 6.1–8.0. Kₘ and Vₘₐₓ for external "Rb⁺ were calculated from both Lineweaver-Burk and Eadie-Hofstee plots. Those values and a calculated mean are shown at each pH. a, calculated Vₘₐₓ; b, calculated Kₘₐₓ.

TABLE III
Influence of ATP on "Rb⁺ exchange

| Exchange | pμmol mg⁻¹ min⁻¹ |
|----------|-----------------|
| Rb⁺, = 0.3 mM |            |
| Control (5.0 mM CDTA) | 344 |
| 1.0 μM ATP           | 326 |
| 10.0 μM ATP           | 332 |
| 100.0 μM ATP          | 255 |
| 1000.0 μM ATP         | 131 |
| Rb⁺, = 3.0 mM |            |
| Control             | 1678 |
| 10.0 μM ATP          | 1404 |
| 100.0 μM ATP         | 795  |
| 1000.0 μM ATP        | 384  |

FIG. 9. Polyacrylamide gel electrophoresis profile of sucrose gradient-resolved proteoliposomes. Proteoliposomes were formed from n-octyl glucoside-solubilized microsomes and resolved by 17-h sedimentation over an 8–46% linear sucrose gradient. G-II was the original microsomal material. Samples were retrieved from the top of the gradient so that subsequent fractions are fractions sedimenting into increasing sucrose density.

the reconstitution procedures employed here yield a mixture of particulate enzyme, liposomes, and proteoliposomes. To resolve this mixture and determine the peptide composition responsible for the transport reactions, the material reconstituted by the n-octyl glucoside-dependent procedure was purified on a sucrose density gradient. The peptide pattern of the sucrose gradient-resolved fractions is shown in Fig. 9. There was a significant loss of contaminating peptides from the original membrane fraction in the lighter gradient fractions. In those fractions a 94-kDa peptide region was the singularly purified peptide region. The gradient resolved fractions were then tested for passive cation exchange and substrate-dependent H⁺ transport activity. As shown in Fig. 10, maximal H⁺ transport and K⁺-stimulated ATPase activities were localized to the lighter fractions. These fractions also contain the maximal Rb⁺/Rb⁺ exchange, phosphoenzyme, and p-nitrophenylphosphatase activity, as well as the purified 94-kDa peptide(s). As shown in Table V, in both cholate and n-octyl glucoside-solubilized preparations, the maximal gradient-resolved ATPase activity represented an approximate 5-fold enrichment over the supernatant enzyme activity, and in the case of n-octyl glucoside, represented an approximate
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FIG. 10. Sucrose gradient resolution of transport activities. Proteoliposomes were formed from n-octyl glucoside-solubilized microsomes in the presence of 50 mM K$_2$SO$_4$ and 10 mM Pipes/Tris, pH 6.1. After resolution over a linear sucrose gradient as in Fig. 9, the activities of each fraction were measured as indicated under "Experimental Procedures." Units utilized for these measurements are: ○, (Mg + K)-ATPase (μmol mg$^{-1}$ h$^{-1}$); Δ, H$^+$ transport (% ΔF/min). ●, Rb$^+$/K$^+$ exchange (pmol/3 min/75-μl aliquot).

| Table V | Partial reactions of reconstituted ATPase|
|---------|----------------------------------------|
| Sample  | ATPase  | pNPPase | E-P  |
| G-II    | 100     | 100     | 100  |
| Cholate |         |         |      |
| Supernatant | 6.8    | 21      | 2.3  |
| Transport peak | 14.5  | 17      |      |
| ATPase peak | 32.2  | 21      |      |
| n-Octyl glucoside |     |         |      |
| Supernatant | 38    | 42      | 39   |
| Transport peak | 119  | 95      | 52   |
| ATPase peak | 188  | 113     | 54   |

doubling of enzyme specific activity in comparison to native vesicles.

DISCUSSION

Until now, reconstitution of transport by the solubilized gastric ATPase, in contrast to the Na,K-ATPase and Ca-ATPase, has been problematic. In this report both cholate and n-octyl glucoside were utilized to solubilize the gastric ATPase prior to reconstitution of transport function. As will be discussed below, this procedure yielded a proteoliposome purified in terms of peptide composition. It should be mentioned that with 2.5% n-octyl glucoside it was important to solubilize the enzyme at acidic pH in the presence of MgATP and reconstitute by liposomal addition prior to detergent removal. This ligand protection was not characteristic of the cholate reconstitution procedure and may reflect a specific interaction of n-octyl glucoside with Mg$^{2+}$ and ATP-binding sites. The singular usefulness of protective ligands for both recovery of supernatant activity and reconstitution of H$^+$ transport, however, did provide evidence for selective reconstituton of the ligand-protected detergent-solubilized ATPase.

The formation of H$^+$ gradients of the magnitude generated by the gastric ATPase probably requires some significant degree of organization of the lipid bilayer. Planar bilayers are often considered to be highly permeable to protons, and if calculated permeabilities were extrapolated to the gastric proteoliposomes, an inefficient transport system would result (29). In separate experiments, the ability of liposomes to maintain artificially imposed pH gradients was determined, and cholesterol was found to be beneficial. Addition of sufficient quantities of this lipid to the reconstitution mixture was also found to improve the ATP-dependent acidification process. Since lipid-associated peptide is undoubtedly present in the detergent extracts utilized, it was not surprising that the nature of phospholipids used for reconstitution of enzyme transport fraction was not critical. In the course of these experiments H$^+$ transport was successfully reconstituted from crude mixtures of soyalectin, lecithin, and mixtures of lecithin, phosphatidylethanolamine, and cholesterol. The lipid composition of gastric membranes is known (30), but it was not found necessary to precisely duplicate this lipid composition when reconstituting transport functions.

Gastric membranes, even when purified by free-flow electrophoresis, contain peptides other than the 94-kDa catalytic subunit region. Thus, it was not certain that all the partial reactions of enzyme activity and transport were a function of the 94-kDa peptide(s). In this study, ATPase activity, active H$^+$ transport, and passive cation exchange were correlated with enrichment of the 94-kDa peptide region. These fractions showed greater than 90% of the Coomassie Blue staining material localized to this region. To ascribe any transport function to a contaminating peptide would require either an extremely active small molecular weight peptide or that it too is approximately 94 kDa. This peptide purification strengthened the conclusion that the observed partial reactions and transport functions of the gastric microsomal preparation were due to the high molecular weight protein.

From a variety of data in gastric microsomes, it had been concluded that the ATPase was responsible for a uniquely electroneutral H$^+$ for K$^+$ exchange (13, 23). Still, it remained possible in gastric microsomes that undetermined shunt pathways could have obscured an electrogenic pump function, as was the case with the Na,K-ATPase (31). Two central properties of active proton transport in gastric microsomes were confirmed in the reconstituted proteoliposomes. Formation of a pH gradient in the presence of an impermeant anion such as SO$_4^{2-}$ is insensitive to protonophores, and $\Delta$ΨH$^+$ can be measured only in the presence of the protonophore. These experiments provide strong evidence that the pump is indeed electroneutral.

In the reconstituted liposomes, H$^+$ transport demonstrated an internal K$^+$ or Rb$^+$ requirement. The demonstration of Mg$^{2+}$-ATP-dependent "Rb" efflux from isotopic equilibrium in the reconstituted system was consistent with that found in native microsomes. This MgATP, not MgADP- or ATP-dependent "Rb" transport could not be explained by selective inhibition of "Rb" uptake, and since it was also protonophore insensitive could not be due to development of a membrane potential.

In contrast to native microsomes, however, the extent and rate of pH formation was greater in sulfate solutions than chloride. This was attributed to the presence of a Cl$^-$ conductance as the protonophore, TCS, induced a rapid dissipation of the H$^+$ gradient in Cl$^-$ but not sulfate solutions. A Cl$^-$ conductance has been described in microsomes obtained from...
stimulated rabbit mucosa (32), but here the proteoliposomes were not sufficiently permeable to K+ that H+ transport could be measured in the absence of K+ equilibration. The reduction in H+ gradient capacity in C1- solutions also implies the presence of a significant H+ conductance in the reconstituted system. In sulfate, the size of the pH gradient that can be generated is considerable. With a vesicular volume of 17 μl/nm protein, and from the equation ΔpH = log Q/1 - Q + log V/μ, where Q is the fractional fluorescence quench of acridine orange and V/μ is the inverse of the fractional vesicular volume, a value of greater than 5 pH units could be calculated. This calculated pH gradient was larger than that obtained in the microsomes, although the rate of intravesicular acidification was considerably slower. This latter phenomenon can be accounted for, at least in part, by the almost 10-fold larger vesicular volume and almost certainly by the smaller number of pump units/liposome.

The reconstituted H,K-ATPase demonstrated comparable rates of vanadate-sensitive cation exchange and active cation efflux. Vanadate sensitivity was used to show that about 70% of the proteoliposomes were oriented with the ATP site facing outward and 30% had the ATP site facing inward. This would lead to underestimation of the active Rb+ efflux but does not affect the conclusion that the ratio of active Rb+ efflux and passive cation exchange are similar, though not equivalent. This near equivalent rate of exchange and active Rb+ transport suggests that the former reaction is not limited by a K+-occluded form of the enzyme.

The model shown in Fig. 11 is a hypothetical scheme to account for the interactions of ATP and Mg2+ with K+-K+ exchange. As depicted, exchange occurs following a conformational change with K+ bound to the cytoplasmic (E1) and/or luminal (E2) binding sites of the ATPase. It is assumed that ATP binding promotes the E1 conformation and PO4- the E2. E(K+) is a putative intermediate form which exists in the membrane, although the rate of intravesicular acidification was considerably slower. This latter phenomenon can be accounted for, at least in part, by the almost 10-fold larger vesicular volume and almost certainly by the smaller number of pump units/liposome.

The reconstituted H,K-ATPase, an E(K+) form does exist in the absence of these ligands the ratio of E1/E2 species. The inhibition of exchange by VO3- is consistent with the stimulation of H2O-exchange by K+, as measured by NMR of H+O3- (33).

Inhibition of exchange by VO3- in a noncompetitive manner suggests enzyme trapping in some E-V O4 form, reducing the number of enzyme units available for exchange.

The relationship observed between Rb+ and H+ concentration in Rb+ exchange was not competitive, appearing instead to be largely noncompetitive. This could account for our inability to observe passive H/K exchange in the absence of other ligands. We may speculate that the physiological role of this would be to prevent the passive leak of the large H+ gradients generated by this ATPase.

A significant aspect of these findings is that reconstitution methods can be combined with gradient procedures and used to purify only those proteoliposomes capable of transport. Along with this, most of the gastric vesicle peptides, apart from the 94-kDa region, were lost. This suggests that similar methods applied to vesicles derived from stimulated mucosa might achieve the necessary purification to allow definition of the peptide(s) responsible for the changes in conductance observed in these preparations.

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Fig. 11. K+?K+ exchange of the H,K-ATPase. A model illustrating the reaction pathway involved in K+?K+ exchange of the H,K-ATPase, in the absence and presence of ATP or Mg2+ and Pi, or Mg2+ and vanadate.